# Studies on Characterization and Growth Control of Sugar-tolerant Yeasts( Dissertation_全文 )

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## INTRODUCTION

Microorganisms which can be alive in environments with high sugar or high salt contents include halophilic bacteria, halotolerant bacteria, halophilic algae, osmotolerant yeasts, and xerophilic fungi (1, 2). Halophilic bacteria had been studied most extensively, as reviewed by some researchers (3, 4).

Since the term "osmophilic" was given by von Richter (5) to the group of yeasts which can grow well in environments with high osmophilic pressures, yeasts showing sugar- and salt-tolerant properties have been traditionally called "osmophilic" or "osmotolerant". However, these terms are not considered to be always accurate, based on the results in the previous studies (6, 7), therefore, it is recommended that they should be designated as "sugar-tolerant" and "salt-tolerant". Although there are no strict definitions on sugar- and salt-tolerant yeasts, sugar-tolerance of yeasts is tested using a medium containing 50% (w/w) glucose in the latest yeast taxonomy (8).

Sugar-tolerant yeasts have been reported to cause the spoilage of intermediate moisture foods such as confectionery, jams, dry fruits, and honey (9, 10). In recent years, some techniques including gas-packaging, oxygen-absorber packaging, and addition of ethanol were developed and widely used in order to prolong the shelf-life of such foods. The technique for removing oxygen in environments effectively inhibits the growth of fungi, but it is not so effective for depressing that of yeasts, which can grow under anaerobic conditions. Therefore, the growth of yeasts is found even in foods packaged without oxygen, resulting in swelling of pouches by generation of carbon dioxide and degradation of food quality by production of alcohol, ethyl acetate, and so on.

These days, flavor and taste, and safety of foods are thought to be important, so development of techniques for depressing the growth of yeasts other than heating and addition of artificial preservatives is increasingly important mainly for preservation of intermediate moisture foods. Therefore, the study on the physiological characteristics of sugar-tolerant yeasts and methods for depressing their growth is needed.

In this study, sugar-tolerant yeasts were isolated and identified. Their characteristics and the effects of controled environmental conditions and various chemical substances on their growth were investigated. Basic findings on the effective methods for growth control of sugar-tolerant yeasts were obtained.

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# Chapter I

## Isolation and identification of sugar-tolerant yeasts

This chapter deals with the isolation and identification of yeasts from confectionery, fruits and processed fruits, honey, molasses, and other related materials.

### I-1. Identification of yeasts isolated

#### MATERIALS AND METHODS

*Samples.* Two hundred and sixty-five samples were used for isolation of yeasts. Twenty-three samples were already fermented. Fifteen samples of confectionery including sponge cake and mizu-yokan (sweet bean curd containing 45% sugar), six samples of fruits, 11 samples of fruit jams, four samples of dried fruits such as raisins and dried dates, six samples of candied fruits, and concentrated coffee were obtained from local food markets and food processing plants. Different kinds of sugars including raw and refined sugar (ten samples) and syrups (nine samples) were obtained from sugar refinery plants and local markets. Molasses (125 samples) was obtained from Japan, Thailand, and Taiwan. Most of 14 samples of honey were obtained directly from some apiaries in Japan. Flowers including azalea, dandelion, and clover (36 samples), orchid nectar (six samples) and cherry tree sap (one sample) were collected at the botanical garden of the University of Tokyo, and 16 samples of soil were obtained from the orchard of the Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries. Three samples of soy sauce and miso (soy paste) were used for the isolation of salt-tolerant yeasts for comparison.

*Isolation of yeasts.* Yeasts were isolated by direct streaking on media containing 25% or 40% (w/w) glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, and 2.5% agar. Enrichment culture was also employed for the isolation of yeasts by repeating subcultures in liquid media containing 25% or 40% (w/w) glucose, 0.5% peptone, 0.3% malt extract, and 0.3% yeast extract. Isolated yeast strains were purified by conventional streaking technique using the same media as those used for isolation.

*Measurement of water activity.* The water activity ($a_w$) of media was measured by electric hygrometry using Hygroskop DT (Rotronic, Switzerland).

*Test of sugar-tolerance and salt-tolerance of the isolates.* Seven kinds of media, YM agar ($a_w$ 0.986), 25% ($a_w$ 0.951), 40% ($a_w$

0.912), or 50% ($a_w$ 0.876) (w/w) glucose agar, different in only glucose concentration from YM agar, and 10% ($a_w$ 0.932), 15% ($a_w$ 0.894), or 18% ($a_w$ 0.860) (w/w) NaCl- YM agar were used for testing sugar-tolerance and salt-tolerance. Actively growing cultures on YM agar were inoculated on to agar plates with a multipoint inoculating apparatus (Kyoritsu Seisakujo, Tokyo, Japan). The plates were tightly closed with rubber bands and incubated at 26°C for 2 weeks, and the diameter of each colony was measured.

*Selection of yeast strains for identification.* Fermentation of glucose, assimilation of maltose, galactose, sucrose, raffinose, lactose, and nitrate, growth in vitamin-free medium, and growth at 30, 37, and 42°C were investigated for all of the strains isolated. The representative yeast strains for identification were selected according to the results of these tests, sugar-tolerance and salt-tolerance, morphology of vegetative cells, appearances of colonies and isolation sources.

*Identification of yeasts.* Methods of identifying yeasts were those described in "The Yeasts, a Taxonomic Study", 3rd ed. (1). The conjugation test was performed by the procedure of Hamamoto et al. (2). DNA base composition (mol% guanine plus cytosine) and the quinone systems were determined for 22 strains. DNA was isolated from the culture at the logarithmic phase according to the procedure of Saito and Miura (3) with some modifications. DNA was spooled around a glass rod to eliminate mitochondrial DNA (4, 5). DNA base composition was determined by reversed-phase high-performance liquid chromatography (HPLC) (6). Quinone was extracted from intact cells and partially purified by thin-layer chromatography by a modification of the method of Yamada et al. (7). Quinone system was determined by HPLC (8).

## RESULTS

The number of the samples from which yeasts were isolated and the number of yeast strains isolated are shown in Table I-1. Three hundred and twenty-four strains were isolated from 140 samples, which was more than half of all the samples used. The ratios of samples from which yeasts were isolated were relatively low in confectionery, fruit jams, sugars, and syrups, but high in fruits, candied fruits, and molasses. The appearances of colonies of the isolates from molasses and honey were similar to each other.

Fig. I-1 shows three growth patterns of isolates at various glucose concentrations. Type 1 includes yeasts that grow only on YM agar or 25% (w/w) glucose medium. Type 2 includes yeasts that

Table I-1 Sources and number of yeast strains isolated.

| Source | Ratio of samples from which yeasts were isolated | Number of strains | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | Direct streak | | | Enrichment | | |
| | | YM[a] | 25% glc [b] | 40% glc [c] | 25% glc | 40% glc | |
| confectionery | 5/15 | 5 | 1 | 1 | /[f] | 1 | 8 |
| fruit | 6/6 | 4 | 6 | 12 | 1 | 7 | 30 |
| fruit jam | 4/11 | 5 | 2 | 4 | 1 | 1 | 13 |
| dried fruit | 2/4 | 1 | 3 | 1 | 2 | 1 | 8 |
| candied fruit | 4/6 | 1 | 4 | 4 | 4 | 4 | 17 |
| concentrated coffee | 1/1 | / | 1 | / | / | / | 1 |
| sugar, syrup | 2/19 | 1 | 2 | 2 | 2 | 1 | 8 |
| molasses | 3/4[d] | 3 | 2 | 9 | 2 | 2 | 18 |
| 〃 | 78/121[e] | / | / | 124 | / | / | 124 |
| honey | 4/14 | 1 | 2 | 10 | 1 | 8 | 22 |
| flower | 12/36 | 2 | 5 | 14 | 7 | 7 | 35 |
| nectar | 2/6 | 0 | 0 | 2 | / | 1 | 3 |
| soil | 13/16 | 3 | 9 | 7 | 5 | 7 | 31 |
| sap | 1/1 | 1 | / | 1 | / | / | 2 |
| soy sauce, miso | 3/3 | 0 | / | 2 | / | 2 | 4 |
| others | 0/2 | 0 | 0 | 0 | / | / | 0 |
| Total | 140/265 | 27 | 37 | 193 | 25 | 42 | 324 |
| | | | 257 | | 67 | | |

[a] YM agar medium containing 1% glucose, 0.5% polypeptone, 0.3% malt extract, 0.3% yeast extract, and 2% agar was also used.
[b] 25% (w/w) glucose medium.
[c] 40% (w/w) glucose medium.
[d] obtained from Japan and Thailand.
[e] obtained from Taiwan.
[f] not tested.

are able to grow on 40% or 50% (w/w) glucose medium as well as YM agar. Type 3 yeasts grow on 25% or 40% (w/w) glucose medium better than on YM agar. In this study, Type 2 and Type 3 yeasts were defined respectively as sugar-tolerant and osmophilic. More than 60% of the isolates belong to Type 2, and only a few strains belong to Type 1. Most of the Type 3 yeast strains were isolated from honey, molasses, soy sauce, and miso. Ninety-nine strains out of 324 were selected for identification on the basis of morphological, biochemical and physiological properties. Identification of 93 strains are described here.

Fifty-four strains out of 93 were identified as belonging to 12 species in eight genera of ascosporogenous yeasts, and 39 strains were identified as belonging to 26 species in five genera of asporogenous yeasts. Major species identified were

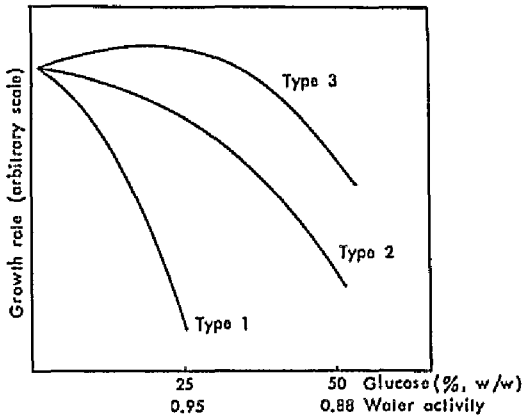


Fig. I-1 Three growth patterns of isolates with regard to glucose concentrations.

Table I-2 Major yeast species identified and sources of isolation.

| Species | Source | | | | | | |
|---|---|---|---|---|---|---|---|
| | Confectionery | Jam | Candied fruit | Sugar or molasses | Honey | Fruit | Soil |
| *Candida apicola* | | | | | | 2 | |
| *Debaryomyces hansenii* | | 2 | | | | 2 | |
| *Hansenula anomala* | 2[a] | 1 | | | | 4 | |
| *Kloeckera apis* | | | | | | 2 | |
| *Saccharomyces cerevisiae* | | | 1 | | | | 3 |
| *Schizosaccharomyces octosporus* | | | | 3 | | | |
| *Schizosaccharomyces pombe* | | | | 1 | | | |
| *Torulaspora delbrueckii* | 1 | | 1 | | | 1 | |
| *Torulaspora globosa* | | | | 1 | | 1 | 1 |
| *Zygosaccharomyces rouxii* | | | 3 | 7 | 6 | | |

[a] Number of strains.

*Zygosaccharomyces rouxii*, *Hansenula anomala*, *Debaryomyces hansenii*, and *Saccharomyces cerevisiae*. All strains of *Z. rouxii* were osmophilic (Type 3). The other species were sugar-tolerant (Type 2).

Table I-2 shows the representative yeast species identified and the sources of isolation. All the isolates from honey were *Z. rouxii*, but various species were isolated from fruits. Generally, the *Zygosaccharomyces* and *Schizosaccharomyces* strains were isolated from the foods with particularly high sugar content such as honey, candied fruits, and molasses. *Z. rouxii* has already been reported to be the predominant yeast in honey (9, 10). *Z. rouxii* and *Zygosaccharomyces bisporus*, which were isolated from molasses, have been reported to be predominant species in unrefined sugar (11).

*Identification of yeasts*

Characteristics of the identified yeasts are shown in Table I-3. Using these characteristics, the strains were identified as species. Standard descriptions of the yeast species are from "The Yeasts," 3rd ed. (1). Sources of isolation are indicated here in parentheses.

*Debaryomyces hansenii* (Zopf) Lodder et Kreger-van Rij

Strains: 7-50A (flower), 22-40B (apple), 23-25A (banana), 67-40A (blueberry sauce), and 68-40A (marmalade)

The properties of these five strains fit the standard species description.

*Hanseniaspora guilliermondii* Pijper

Strain: 25-CA (kiwi fruit)

The properties of this strain almost fit the standard species description. It did not grow on 50% (w/w) glucose- yeast extract agar.

*Hansenula anomala* (Hansen) H. et P. Sydow

Strains: 24-CA, 24-40A, and 25-50A (kiwi fruit), 126-25A (pineapple), 148-40A (jam), 150-40A and 150-40B (sponge cake), and 157-25A (concentrated coffee)

The properties of these strains fit the standard species description.

*Pichia membranaefaciens* Hansen

Strain: 114-CA (soil)

The properties of this strain matched the standard species description. It had 41.9 mol% guanine plus cytosine in the DNA, and ubiquinone Q-7.

*Pichia ohmeri* (Etchells et Bell) Kreger-van Rij

Strain: 19-40A (flower)

The properties of this strain almost matched the standard species description. It assimilated soluble starch.

*Saccharomyces cerevisiae* Meyen ex Hansen

Strains: 83-25A, 124-40A and 124-40EA (soil), and 89-25A (candied apple)

The properties of these strains fit the standard species description. Strain 89-25A can be identified as *S. cerevisiae* according to "The Yeasts", 2nd ed. (12), and the others as *S. chevalieri*.

*Schizosaccharomyces octosporus* Beijerinck

Strains: 37-CB and 37-40B (molasses)

The properties of these strains almost matched the standard species description. Neither strain grew at 37°C.

*Schizosaccharomyces pombe* Lindner

Strains: 57-50B (molasses) and 128-40A (raisin)

The properties of these strains fit the standard species

description.

*Torulaspora delbrueckii* (Lindner) Lindner

Strains: 89-40EA (candied apple), 126-50B (pineapple), and 154-CA (mizuyokan)

The properties of these strains matched the standard species description. Strains 89-40EA and 126-50B did not assimilate maltose, while strain 154-CA assimilated and fermented it.

*Torulaspora globosa* (Klöcker) van der Walt et Johannsen

Strains: 26-40A (papaya), 57-25A (molasses), and 77-40A (soil)

The properties of these strains fit the standard species description.

*Zygosaccharomyces bisporus* Naganishi

Strain: 153-40A (molasses)

The properties of this strain matched the standard species description. According to "The Yeasts" (1), the morphological, biochemical and physiological properties of *Zygosaccharomyces bailii*, *Z. bisporus* and *Z. rouxii* are similar to each other. van der Walt (13) pointed out that delimitation of these species on the basis of utilization of sugars is uncertain because only a few sugars are utilized by them. *Z. bailii* has been reported to be very resistant to preservatives such as benzoic acid, sorbic acid, and acetic acid (14, 15). Growth on media containing 400 mg/l of benzoic acid, 300 mg/l of sorbic acid, or 1% acetic acid provides a simple and rapid identification test to discriminate these species (1). Strain 153-40A did not grow in the presence of 400 mg/l of benzoic acid and 300 mg/l of sorbic acid, but it tolerated 1% acetic acid. On the basis of these properties, we identified it as *Z. bisporus*.

*Zygosaccharomyces rouxii* (Boutroux) Yarrow

Strains: 38-40A, 38-40EA, 45-40A, 45-50B, 46-50A, and 46-50B (honey), 52-CA, 52-CB, 52-25A, 52-40A, 151-40A, and 151-40B (molasses), 56-40A (washed sugar), 88-25A and 88-40A (candied cherry), 90-25EA (candied orange), 86-A, 86-B, 86-50A, and 149-A (soy sauce), and 87-A, 87-B, and 87-50A (miso)

The properties of these strains almost fit the standard species description. None of the isolates assimilated D-ribose, and approximately half assimilated D-xylose weakly. They were not resistant to benzoic acid, sorbic acid, or acetic acid. The DNA base composition of strain 38-40A was 38.4 mol% guanine plus cytosine. From these properties, they were identified as *Z. rouxii*.

*Candida apicola* (Hajsig) Meyer et Yarrow

Strains: 22-40A (apple) and 23-40A (banana)

The properties of these strains almost fit the standard species description. However, fermentation of raffinose,

Table I-3 Characteristics of yeasts isolated from

| Species and strain No. | Fermentation of glucose | Assimilation | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Galactose | L-Sorbose | Sucrose | Maltose | Cellobiose | Trehalose | Lactose | Melibiose | Raffinose | Melezitose | Inulin | Soluble starch | D-Xylose | L-Arabinose | D-Arabinose |
| Ascosporogenous yeasts | | | | | | | | | | | | | | | | |
| *Debaryomyces hansenii* | | | | | | | | | | | | | | | | |
| 7-50A, 22-40B, 67-40A, 68-40A | − | + | + | + | + | + | + | W | +[a] | + | + | − | + | + | + | − |
| 23-25A | VW | + | + | + | + | + | + | + | + | + | + | − | + | + | + | − |
| *Hanseniaspora guilliermondii* | | | | | | | | | | | | | | | | |
| 25-CA | + | − | − | − | − | + | − | − | − | − | − | − | − | − | − | − |
| *Hansenula anomala* | | | | | | | | | | | | | | | | |
| 24-CA, 126-25A | + | + | − | + | + | + | + | − | − | + | + | − | + | − | − | − |
| 24-40A | + | + | − | + | + | + | + | − | − | − | + | − | + | W | − | − |
| 25-50A, 148-40A, 150-40A | + | + | − | + | + | + | + | − | − | + | + | − | + | +[a] | − | − |
| 150-40B | + | W | − | + | + | + | + | − | − | − | + | − | + | W | − | − |
| 157-25A | + | + | − | + | + | + | + | − | − | + | + | − | + | W | − | − |
| *Pichia membranaefaciens* | | | | | | | | | | | | | | | | |
| 114-CA | − | − | + | − | − | − | − | − | − | − | − | − | − | − | − | − |
| *Pichia ohmeri* | | | | | | | | | | | | | | | | |
| 19-40A | + | + | + | + | + | + | + | − | − | + | − | + | + | − | − | − |
| *Saccharomyces cerevisiae* | | | | | | | | | | | | | | | | |
| 83-25A | + | + | − | + | − | − | + | − | − | W | + | + | W | − | − | − |
| 89-25A | + | + | − | + | + | − | + | − | − | + | − | − | − | − | − | − |
| 124-40A | + | + | − | + | − | W | + | − | − | + | − | − | + | − | − | − |
| 124-40EA | + | + | − | + | W | − | + | − | − | + | − | − | − | − | − | − |
| *Schizosaccharomyces octosporus* | | | | | | | | | | | | | | | | |
| 37-CB, 37-40B | + | − | − | − | − | − | − | − | − | − | − | W | − | − | − | − |
| *Schizosaccharomyces pombe* | | | | | | | | | | | | | | | | |
| 57-50B, 128-40A | + | − | − | + | + | − | − | − | − | + | − | − | − | − | − | − |
| *Torulaspora delbrueckii* | | | | | | | | | | | | | | | | |
| 89-40EA, 126-50B | + | − | − | + | − | − | + | − | − | + | − | + | − | − | − | − |
| 154-CA | + | − | − | + | + | − | + | − | − | + | − | + | − | − | − | − |
| *Torulaspora globosa* | | | | | | | | | | | | | | | | |
| 26-40A, 57-25A, 77-40A | + | − | W[b] | + | − | − | + | − | − | + | − | + | − | − | − | − |
| *Zygosaccharomyces bisporus* 153-40A | + | W | − | − | − | − | − | − | − | − | − | − | − | − | − | − |

+: positive, −: negative, W: weak, VW: very weak.

high-sugar foods and related materials.

| D-Ribose | L-Rhamnose | Glycerol | Erythritol | Ribitol | Galactitol | D-Mannitol | D-Glucitol | α-Me-D-glucoside | Salicin | DL-Lactic acid | Succinic acid | Citric acid | Inositol | Nitrate | Growth without vitamins | Growth at 37°C | Formation of ascospores | GC content (mol %) | Co-Q system | Type for sugar-tolerance |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| − | − | + | + | + | − | + | + | + | + | W[b] | + | − | − | − | − | − | + | | | 2 |
| − | − | + | + | + | − | + | + | + | + | − | + | + | − | − | − | + | + | | | 2 |
| − | − | − | − | − | − | − | − | − | + | − | − | − | − | − | − | + | + | | | 1 |
| − | − | + | + | − | − | + | − | + | + | + | + | + | − | + | + | + | + | | | 2 |
| − | − | + | + | − | − | + | − | + | + | + | + | + | − | + | + | + | + | | | 2 |
| − | − | + | + | − | − | + | − | + | + | + | + | + | − | + | + | + | + | | | 2 |
| − | − | + | + | − | − | + | W | + | + | + | + | + | − | + | + | − | + | | | 2 |
| W | − | + | + | − | − | + | W | + | + | + | + | + | − | + | + | − | + | | | 2 |
| − | − | + | − | − | − | − | − | − | − | + | + | − | − | − | + | + | + | 41.9 | $Q_7$ | 1 |
| − | − | + | − | + | − | + | + | + | + | − | + | + | − | − | − | + | + | | | 2 |
| − | − | W | − | − | − | − | − | + | − | W | − | − | − | − | − | + | + | | | 2 |
| − | − | − | − | − | − | − | − | + | W | − | − | − | − | − | + | + | + | | | 2 |
| − | − | − | − | − | − | − | − | + | − | − | − | − | − | − | − | + | + | | | 2 |
| − | − | + | − | − | − | + | + | − | − | − | − | − | − | − | − | + | + | | | 2 |
| − | − | − | − | − | − | − | − | + | − | − | − | − | − | − | − | − | + | | | 2 |
| − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | + | + | | | 2 |
| − | − | + | − | − | − | + | + | W | − | + | − | − | − | − | + | − | + | | | 2 |
| − | − | + | − | − | − | + | + | W | − | + | − | − | − | − | + | − | + | | | 2 |
| − | − | + | − | − | − | + | + | − | − | + | − | − | − | − | + | + | + | | | 2 |
| − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | + | | | 3 |

[a] sometimes weak. [b] sometimes negative.

Table I-3

| Species and strain No. | Fermentation of glucose | Assimilation: Galactose | L-Sorbose | Sucrose | Maltose | Cellobiose | Trehalose | Lactose | Melibiose | Raffinose | Melezitose | Inulin | Soluble starch | D-Xylose | L-Arabinose | D-Arabinose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Zygosaccharomyces rouxii* | | | | | | | | | | | | | | | | |
| 38-40A, 38-40EA, 45-50B, 52-CB, 88-25A | + | − | − | − | +[a] | − | W[b] | − | − | − | − | − | − | W | − | − |
| 45-50A, 46-50A, 46-50B, 52-CA, 90-25EA | + | +[a] | − | +[a] | +[a] | W[b] | − | − | − | − | − | − | − | W | − | − |
| 52-25A, 52-40A | + | − | − | − | − | − | − | − | − | − | − | − | − | W[b] | − | − |
| 56-40A, 87-50A, 88-40A, 149-A | + | W[b] | − | − | +[a] | − | − | − | − | − | − | − | − | − | − | − |
| 86-A, 86-B, 86-50A, 87-B | + | W | − | − | + | − | + | − | − | − | − | − | − | − | − | − |
| 87-A | + | W | − | − | + | − | − | − | − | − | − | − | − | − | − | − |
| 151-40A, 151-40B | + | + | − | W | + | − | − | − | − | − | − | − | − | − | − | − |
| Asporogenous yeasts | | | | | | | | | | | | | | | | |
| *Candida apicola* | | | | | | | | | | | | | | | | |
| 22-40A, 23-40A | + | − | + | + | − | − | − | − | − | + | − | − | − | − | − | − |
| *Candida bombi* | | | | | | | | | | | | | | | | |
| 10-25EA, 19-25A | + | − | + | + | − | − | − | − | − | + | − | − | − | − | − | − |
| *Candida bombicola* | | | | | | | | | | | | | | | | |
| 11-40EA, 34-25A, 34-40B | + | + | + | + | − | − | − | − | − | + | − | − | − | − | − | − |
| *Candida dattila* | | | | | | | | | | | | | | | | |
| 128-25A | + | + | − | + | + | − | + | − | W | + | + | + | − | W | − | − |
| *Candida famata* | | | | | | | | | | | | | | | | |
| 147-40A | + | + | + | + | + | + | + | − | − | + | + | − | + | + | − | − |
| *Candida guilliermondii* | | | | | | | | | | | | | | | | |
| 22-25A | + | + | + | + | + | + | + | − | + | + | + | + | − | + | + | + |
| *Candida intermedia* | | | | | | | | | | | | | | | | |
| 147-CA | + | + | + | + | + | + | + | + | − | + | + | W | + | + | − | + |
| *Candida krusei* | | | | | | | | | | | | | | | | |
| 156-25A | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| *Candida lactiscondensi* | | | | | | | | | | | | | | | | |
| 91-40A, 158-25A | + | − | − | + | − | − | − | − | − | W | − | − | − | − | − | − |
| *Candida lusitaniae* | | | | | | | | | | | | | | | | |
| 91-CA | + | + | + | + | + | + | + | − | − | − | + | − | − | + | − | − |
| *Candida mannitofaciens* | | | | | | | | | | | | | | | | |
| 152-40A | + | + | − | + | + | + | + | + | W | W | − | W | − | + | W | − |

+: positive, −: negative, W: weak, VW: very weak.

(Continued).

| D-Ribose | L-Rhamnose | Glycerol | Erythritol | Ribitol | Galactitol | D-Mannitol | D-Glucitol | α-Me-D-Glucoside | Salicin | DL-Lactic acid | Succinic acid | Citric acid | Inositol | Nitrate | Growth without vitamins | Growth at 37°C | Formation of ascospores | GC content (mol %) | Co-Q system | Type for sugar-tolerance |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| − | − | + | − | W | − | + | + | − | − | − | − | − | − | − | − | − | + | 38.4 (38-40A) | | 3 |
| − | − | + | − | W | − | + | + | − | − | − | − | − | − | − | − | − | + | | | 3 |
| − | − | + | − | −/+ | − | − | + | − | − | − | − | − | − | − | − | − | + | | | 3 |
| − | − | + | − | − | − | W | +[a] | − | − | − | − | − | − | − | − | − | + | | | 3 |
| − | − | + | − | − | − | + | +[a] | − | − | − | − | − | − | − | W[b] | − | + | | | 3 |
| − | − | + | − | − | − | − | W | − | − | − | − | − | − | − | W | − | + | | | 3 |
| − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | + | | $Q_6$ | 3 |
| W | − | + | − | − | − | + | + | − | − | − | W | − | − | − | − | − | − | 45.0 44.9 | | 2 |
| W | − | + | − | − | − | + | + | − | − | − | W | + | − | − | − | − | − | 46.4 (19-24A) | | 2 |
| − | − | + | − | − | − | + | + | − | − | − | + | −[a] | − | − | − | − | − | 47.5 (34-25A) | | 2 |
| − | − | + | − | + | − | + | + | + | − | − | W | − | − | − | − | W | − | | $Q_6$ | 2 |
| − | − | + | − | + | − | + | + | W | + | + | + | + | − | − | − | − | − | 36.2 | $Q_9$ | 2 |
| + | − | + | − | + | + | + | + | + | W | + | + | + | − | − | + | + | − | 42.9 | $Q_9$ | 2 |
| − | + | + | − | + | + | + | + | + | + | − | + | − | − | − | − | − | − | 43.6 | | 2 |
| − | − | + | − | − | − | − | − | − | − | + | + | + | − | − | + | + | − | | | 2 |
| − | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | − | − | | | 3 |
| W | + | + | − | + | − | + | + | + | + | + | + | − | − | − | − | + | − | | $Q_8$ | 2 |
| W | − | + | − | − | − | W | − | W | + | − | W | − | − | + | − | − | − | 45.4 | $Q_9$ | 2 |

[a] sometimes weak. [b] sometimes negative.

Table I-3

| Species and strain No. | Fermentation of glucose | Assimilation | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Galactose | L-Sorbose | Sucrose | Maltose | Cellobiose | Trehalose | Lactose | Melibiose | Raffinose | Melezitose | Inulin | Soluble starch | D-Xylose | L-Arabinose | D-Arabinose |
| *Candida nodaensis* | | | | | | | | | | | | | | | | |
| 24-60A | + | + | + | − | + | − | − | − | − | − | − | − | − | − | − | − |
| *Candida oregonensis* | | | | | | | | | | | | | | | | |
| 107-40A | + | − | + | + | + | + | + | − | − | − | + | − | + | + | − | − |
| *Candida silvatica* | | | | | | | | | | | | | | | | |
| 68-CA | − | − | + | − | − | − | + | − | − | − | − | − | − | − | − | − |
| *Candida tropicalis* | | | | | | | | | | | | | | | | |
| 118-40A | + | + | + | + | + | + | + | − | − | − | + | − | + | + | + | − |
| *Candida versatilis* | | | | | | | | | | | | | | | | |
| 24-60B | + | + | − | + | + | + | + | − | − | − | − | − | − | + | − | − |
| *Candida* sp. | | | | | | | | | | | | | | | | |
| 23-40B | + | − | + | + | + | + | + | − | − | − | + | − | − | + | − | + |
| *Candida* sp. | | | | | | | | | | | | | | | | |
| 105-40A | + | − | + | + | − | − | W | − | − | + | − | − | − | − | − | − |
| *Cryptococcus laurentii* | | | | | | | | | | | | | | | | |
| 10-25A | − | + | − | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *Cryptococcus neoformans* | | | | | | | | | | | | | | | | |
| 22-CA | − | + | − | + | + | + | + | − | − | + | + | − | + | + | + | + |
| *Kloeckera apiculata* | | | | | | | | | | | | | | | | |
| 115-CA | + | − | − | − | − | + | − | − | − | − | − | − | − | − | − | − |
| *Kloeckera apis* | | | | | | | | | | | | | | | | |
| 26-50A, 126-CB | + | − | − | − | − | + | − | − | − | − | − | − | − | − | − | − |
| *Rhodotorula glutinis* | | | | | | | | | | | | | | | | |
| 13-40B, 32-40A | − | + | + | + | + | + | + | − | − | + | + | W | + | + | + | + |
| 13-40A, 32-40B, 35-25EA | − | + | + | + | + | + | + | − | − | + | + | + | +[a] | + | − | + |
| *Rhodotorula minuta* | | | | | | | | | | | | | | | | |
| 19-CA | − | − | + | + | − | + | + | + | − | − | + | − | − | + | − | − |
| *Rhodotorula rubra* | | | | | | | | | | | | | | | | |
| 70-25A | − | + | + | + | + | W | + | − | − | + | + | − | W | + | − | + |
| 98-40A | − | W | + | + | W | + | + | − | − | + | − | − | − | + | + | + |
| *Sporobolomyces roseus* | | | | | | | | | | | | | | | | |
| 11-25A | − | W | + | + | + | + | + | − | − | + | + | + | + | + | + | + |
| 65-CA | − | − | + | + | + | + | + | − | − | + | + | + | + | W | W | + |
| 115-25A | − | W | + | + | + | + | + | − | − | + | + | + | + | + | − | + |

+: positive, −: negative, W: weak, VW: very weak.

(Continued).

| D-Ribose | L-Rhamnose | Glycerol | Erythritol | Ribitol | Galactitol | D-Mannitol | D-Glucitol | α-Me-D-Glucoside | Salicin | DL-Lactic acid | Succinic acid | Citric acid | Inositol | Nitrate | Growth without vitamins | Growth at 37°C | Formation of ascospores | GC content (mol %) | Co-Q system | Type for sugar-tolerance |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| W | − | + | − | − | − | + | + | − | − | − | − | + | − | + | − | − | − | 49.6 | $Q_9$ | 2 |
| W | − | + | − | + | − | + | + | + | + | − | + | − | − | − | − | − | − | 46.6 | $Q_9$ | 2 |
| − | − | + | − | + | − | + | + | − | − | − | + | − | − | − | − | − | − | 52.9 | $Q_8$ | 2 |
| W | − | W | − | + | − | + | + | + | − | − | + | + | − | − | − | + | − | 36.9 | | 2 |
| W | − | + | − | − | − | + | − | W | + | − | + | − | − | + | − | − | − | 43.4 | $Q_9$ | 2 |
| W | − | W | − | W | − | + | + | + | W | − | + | + | − | − | − | + | − | 48.5 | $Q_9$ | 2 |
| − | − | + | − | − | − | W | + | − | − | − | W | + | − | − | − | + | − | 48.6 | | 2 |
| + | + | W | W | + | + | + | + | + | + | − | + | + | + | − | + | − | − | | | 1 |
| + | + | − | + | + | − | + | + | + | + | + | + | − | + | − | + | − | − | 47.7 | $Q_{10}$ | 1 |
| − | − | − | − | − | − | − | − | − | + | − | − | − | − | − | − | − | − | | | 1 |
| − | − | − | − | − | − | − | − | − | + | − | − | − | − | − | − | + | − | | | 2 |
| + | − | + | − | + | + | + | + | − | + | − | + | + | − | + | + | − | − | | | 2 |
| + | + | + | − | + | − | + | + | + | + | − | + | + | − | + | + | − | − | | | 2 |
| W | − | + | − | + | − | + | + | + | + | W | + | − | − | − | − | − | − | 50.2 | | 1 |
| + | − | + | − | + | − | + | + | − | W | − | + | + | − | − | − | − | − | | | 2 |
| + | − | + | − | + | W | + | W | − | − | − | + | + | − | − | − | − | − | | | 2 |
| + | − | + | − | + | − | + | + | + | + | − | + | − | − | + | + | − | − | | | 2 |
| + | − | + | − | + | − | + | + | + | + | + | + | − | − | + | + | − | − | | | 2 |
| + | − | + | − | W | − | + | + | + | + | − | + | + | − | + | + | − | − | | | 2 |

[a] sometimes weak. [b] sometimes negative.

assimilation of D-ribose and succinic acid, and growth at 37°C did not fit. The DNA base composition of strains 22-40A and 23-40A was 45.0 and 44.9 mol% guanine plus cytosine, respectively, and strain 22-40A had ubiquinone Q-9.

*Candida bombi* Montrocher

Strains: 10-25EA and 19-25A (flower)

The properties of these strains almost matched the standard species description. These strains did not ferment raffinose, they assimilated D-ribose weakly, and they did not grow at 37°C. The DNA base composition of strain 19-25A was 46.4 mol% guanine plus cytosine and it had ubiquinone Q-9.

*Candida bombicola* (Spencer, Gorin et Tulloch) Meyer et Yarrow

Strains: 34-25A, 34-40B, and 11-40EA (flower)

The properties of these strains almost matched the standard species description. They did not grow at 37°C. Strain 34-25A had 47.5 mol% guanine plus cytosine in the DNA, and ubiquinone Q-9.

*Candida dattila* (Kluyver) Meyer et Yarrow

Strain: 128-25A (raisin)

The properties of this strain almost fit the standard species description. Its assimilation of D-xylose did not conform. It had ubiquinone Q-6.

*Candida famata* (Harrison) Meyer et Yarrow

Strain: 147-40A (sap of cherry tree)

The properties of this strain almost fit the standard species description. Its fermentation of galactose and assimilation of L-arabinose did not agree with the description. It had 36.2 mol% guanine plus cytosine in its DNA, and had ubiquinone Q-9.

*Candida guilliermondii* (Castellani) Langeron et Guerra

Strain: 22-25A (apple)

The properties of this strain almost matched the standard species description. Its growth in vitamin-free medium did not agree with the description. This strain had 42.9 mol% guanine plus cytosine in its DNA, and had ubiquinone Q-9.

*Candida intermedia* (Ciferri et Ashford) Langeron et Guerra

Strain: 147-CA (sap of cherry tree)

The properties of this strain almost matched the standard species description. Assimilation of D-arabinose, glycerol, and galactitol did not match the description. This strain had 43.6 mol% guanine plus cytosine in its DNA.

*Candida krusei* (Castellani) Berkhout

Strain: 156-25A (grape)

The properties of this strain fit the standard species description.

*Candida lactiscondensi* (Hammer) Meyer et Yarrow

Strains: 91-40A (angelica) and 158-25A (candied bean)

The properties of these strains fit the standard species description.

*Candida lusitaniae* van Uden et do Carmo-Sousa

Strain: 91-CA (angelica)

The properties of this strain almost fit the standard species description. Growth in vitamin-free medium did not fit. This strain had ubiquinone Q-8. Formation of ascospores by conjugation of this strain and either of the mating type strains of *Clavinispora lusitaniae* has not yet been observed.

*Candida mannitofaciens* (Onishi et Suzuki) Meyer et Yarrow

Strain: 152-40A (molasses)

The properties of this strain almost matched the standard species description. Assimilation of lactose did not fit. It had 45.4 mol% guanine plus cytosine in its DNA, and had ubiquinone Q-9.

*Candida nodaensis* Yarrow et Menna

Strain: 24-60A (kiwi fruit)

The properties of this strain fit the standard species description. It had 49.6 mol% guanine plus cytosine in its DNA, and had ubiquinone Q-9.

*Candida oregonensis* Phaff et do Carmo-Sousa

Strain: 107-40A (flower)

The properties of this strain almost fit the standard species description. Assimilation of L-sorbose, L-rhamnose, and glycerol did not fit. It had 46.6 mol% guanine plus cytosine in its DNA, and had ubiquinone Q-9.

*Candida silvatica* (van der Walt, van der Klift et Scott) Meyer et Yarrow

Strain: 68-CA (marmalade)

The properties of this strain almost fit the standard species description. Assimilation of L-sorbose and DL-lactic acid and growth at 37°C did not fit. It had 52.9 mol% guanine plus cytosine in its DNA, and ubiquinone Q-9. It was neither sugar-tolerant nor osmophilic.

*Candida tropicalis* (Castellani) Berkhout

Strain: 118-40A (lemon cake)

The properties of this strain almost matched the standard species description. Fermentation of maltose and raffinose did not match and it had 36.9 mol% guanine plus cytosine in its DNA, and it had ubiquinone Q-9.

*Candida versatilis* (Etchells et Bell) Meyer et Yarrow

Strain: 24-60B (kiwi fruit)

The properties of this strain almost fit the standard species description. Assimilation of D-xylose, D-glucitol, and succinic acid did not fit. It had 43.4 mol% guanine plus cytosine in its

DNA, and had ubiquinone Q-9.

*Candida* sp.

Strain: 23-40B (banana)

Although the properties of this strain were similar to the standard description of *Candida musae*, it assimilated cellobiose, D-arabinose, and salicin, and grew at 37°C. This strain had 48.5 mol% guanine plus cytosine in its DNA, and had ubiquinone Q-9. It was not identified as belonging to any species reported to date.

*Candida* sp.

Strain: 105-40A (flower)

Although the properties of this strain almost matched the standard description of *C. bombi*, it fermented raffinose very weakly, and had 48.6 mol% guanine plus cytosine in its DNA. This was 2.2 mol% higher than *C. bombi* 19-25A. This strain was not identified as belonging to any species reported to date.

*Cryptococcus laurentii* (Kufferath) Skinner

Strain: 10-25A (flower)

The properties of this strain fit the standard species description. Starch production was positive. This strain was neither sugar-tolerant nor osmophilic.

*Cryptococcus neoformans* (Sanfelice) Vuillemin

Strain: 22-CA (apple)

The properties of this strain almost matched the standard species description. Growth in vitamin-free medium and growth at 37°C did not match. This strain had 47.7 mol% guanine plus cytosine in its DNA, and had ubiquinone Q-10. Starch production was positive. It was neither sugar-tolerant nor osmophilic. Although most of the strains belonging to *C. neoformans* were isolated from clinical sources, non-pathogenic strains such as the type strain of this species and another strain isolated from fruit have also been reported (1, 16).

*Kloeckera apiculata* (Reess emend. Klöcker) Janke

Strain: 115-CA (soil)

The properties of this strain fit the standard species description. It was neither sugar-tolerant nor osmophilic.

*Kloeckera apis* Lavie ex Smith, Simione et Meyer

Strains: 26-50A (papaya) and 126-CB (pineapple)

The properties of these strains matched the standard species description.

*Rhodotorula glutinis* (Fresenius) Harrison

Strains: 13-40A, 13-40B, 32-40A, 32-40B, and 35-25EA (flower)

The properties of these strains matched the standard species description. These strains were classified into two groups according to the assimilation of L-arabinose, L-rhamnose, galactitol, and α-methyl-D-glucoside: Two strains, 13-40B and

32-40A, assimilated L-arabinose and galactitol. The other strains, 13-40A, 32-40B, and 35-25EA, assimilated L-rhamnose and α-methyl-D-glucoside. A mating test has not yet been performed.

*Rhodotorula minuta* (Saito) Harrison

Strain: 19-CA (flower)

The properties of this strain matched the standard species description. The DNA base composition was 50.2 mol% guanine plus cytosine. This strain was neither sugar-tolerant nor osmophilic.

*Rhodotorula rubra* (Demme) Lodder

Strains: 70-25A (jam) and 98-40A (nectar)

The properties of these strains matched the standard species description.

*Sporobolomyces roseus* Kluyver et van Niel

Strains: 11-25A (flower), 65-CA (dried date), and 115-25A (soil)

The properties of these strains matched the standard species description. Strains 65-CA and 115-25A grew at 30°C, while strain 11-25A did not. Formation of ballistospores was observed on corn meal agar.

## DISCUSSION

The results of identification indicate that sugar-tolerant yeasts are widely distributed in various genera. Ascosporogenous yeasts predominate. Fermentative yeasts constituted approximately 80% of the isolates. The relatively small number of strains isolated from confectionery and fruit jams may be due to heating during processing. It is interesting that strains of *C. bombi, C. bombicola, C. mannitofaciens, C. nodaensis, C. oregonensis, C. silvatica,* and *K. apis* were isolated from high-sugar foods, since a few strains of these species have been isolated to date.

More than half of the isolates did not grow on the media containing 50% to 60% (w/w) glucose. It is suggested that media containing 25% to 40% (w/w) glucose may be more suitable for isolating yeasts from high-sugar foods than media containing 50% or 60% (w/w) glucose used in tests of sugar-tolerance (1). Such 25-40% (w/w) glucose media may also be more suitable for practical tests of sugar-tolerance.

Although concentration of sugar or salt in media is usually expressed as percent (w/w) (1, 17) or per cent (w/v) (17, 18), these expressions are likely to be confused. In discussions of sugar-tolerance or salt-tolerance of microorganisms, the $a_w$ of the media would be preferable to percentage of sugar or salt.

The fact that a large number of isolates fermented sugars and produced gas suggests pouch swelling and odor production in gas-packaged foods and vacuum-packaged foods. Further study on the

growth of such yeasts in the presence of high concentrations of sugar is required to develop methods for preventing the spoilage of high-sugar foods.

## I-2. Four new yeast species belonging to the genus *Candida*

In addition to 93 strains described in I-1, five undescribed osmophilic or sugar-tolerant yeast strains were also isolated from brown sugar made in Taiwan, from sponge cake, and from dandelion, azalea, and blueberry flowers. They were identified as four new species in the genus *Candida*, *Candida glucosophila*, *Candida dulciaminis*, *Candida floricola*, and *Candida vaccinii*.

### MATERIALS AND METHODS

*Strains*. Five strains were studied: Strain 29-25A from brown sugar made in Taiwan, strain 155-CA from sponge cake, strain 20-50A from dandelion flowers, strain 34-25EA from azalea flowers, and strain 10-50A from blueberry flowers. *Candida halonitratophila* IFO 1595 (CBS 5240) was also used for comparison.

*Identification methods*. Morphological, physiological, and biochemical characteristics were examined by the methods described in "The Yeasts, a Taxonomic Study", 3rd ed. (1). For strain 29-25A, media containing 25% (w/w) glucose or 10% (w/v) NaCl were used. Extracellular deoxyribonuclease (DNase) activity was determined according to the procedure of Sen and Komagata (19). DNA base composition (guanine plus cytosine mol%) and the quinone systems were determined according to the procedures described in I-1. Unless otherwise stated, all tests were carried out with the cultures incubated at 25°C. "Yeasts, Characteristics and Identification" (20) was also used for identification of the isolates.

*Test of sugar-tolerance*. Five kinds of media, YM agar ($a_w$ 0.987) and 25% ($a_w$ 0.944), 40% ($a_w$ 0.910), 50% ($a_w$ 0.882), or 60% ($a_w$ 0.826) (w/w) glucose agar, different in only glucose concentration from YM agar, were used. Actively growing cultures on YM agar were inoculated onto the agar plates as described in I-1. After incubation at 25°C for 3 weeks, the diameters of two colonies were measured, and an average value was recorded.

### RESULTS AND DISCUSSION

Table I-4 Growth of the five isolates of new *Candida* species and three strains of known species in the presence of various concentrations of glucose.

| Species and strains | Concentration of glucose (%, w/w) | | | | |
|---|---|---|---|---|---|
| | 1 | 25 | 40 | 50 | 60 |
| *Candida glucosophila* | | | | | |
| 29-25A | — | 4.8 | 3.2 | 2.1 | 1.0 |
| *Candida dulciaminis* | | | | | |
| 155-CA | 16.5 | 10.4 | 4.8 | 2.8 | 0.2 |
| *Candida floricola* | | | | | |
| 20-50A | 20.0 | 12.8 | 7.7 | 3.6 | 0.2 |
| 34-25EA | 20.2 | 13.0 | 7.9 | 3.6 | 0.2 |
| *Candida vaccinii* | | | | | |
| 10-50A | 17.6 | 13.9 | 7.2 | 2.6 | 0.2 |
| *Pichia membranaefaciens* | | | | | |
| 114-CA | 12.4 | 4.8 | — | — | — |
| *Rhodotorula glutinis* | | | | | |
| 13-40B | 13.6 | 4.0 | 2.3 | — | — |
| *Zygosaccharomyces rouxii* | | | | | |
| 88-40A | 5.4 | 7.3 | 4.8 | 3.0 | 0.7 |

—: No growth.
Numerals represent diameters (mm) of colonies measured after three weeks' incubation.

Descriptions of four new species are given below. Table I-4 shows the growth of five isolates in the presence of various concentrations of glucose. The growth of *P. membranaefaciens* 114-CA (non sugar-tolerant), *R. glutinis* 13-40B (sugar-tolerant), and *Z. rouxii* 88-40A (osmophilic) described in I-1 is also shown for comparison. The isolate 29-25A does not grow on YM agar and grows best on 25% (w/w) glucose- YM agar. This means this strain is obligately osmophilic. The other four strains can grow on 60% (w/w) glucose- YM agar and are sugar-tolerant yeasts.

1. *Candida glucosophila* Tokuoka, Ishitani, Goto et Komagata sp. nov.

In liquido "YM" cum 25% (w/w) glucoso, post dies 7 ad 25°C, cellulae globosae aut subglobosae, 4.2-7.0 x 4.2-7.3 μm, singulae, binae, in catenis ramosis brevis, vel in fasciculis. In agaro "YM" cum 25% (w/w) glucoso, post unum mensem ad 25°C, cultura cremea, opaca, sicca, laevis, margine integra. Pseudomycelium nullum. Ascosporae nullae, ballistosporae nullae et teliosporae nullae. Non nisi glucosum fermentatur. Glucosum et D-xylosum (exiguum) et glycerolum (exiguum) assimilantur at non galactosum, L-sorbosum, sucrosum, maltosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinosum, melezitosum,

inulinum, amylum solbile, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, erythritolum, ribitolum, galactitolum, D-mannitolum, D-glucitolum, alpha-methyl-D-glucosidum, salicinum, acidum DL-lacticum, acidum succinicum, acidum citicum nec inositolum. Kalium nitricum assimilatur. Ad crescentiam vitaminae externae necessariae sunt. Nullum incrementum in agaro "YM" cum 1% glucoso, optimum incrementum in agaro "YM" cum 25% (w/w) glucoso. Ureum non hydrolysatur. Diazonium caeruleum B: negativum. Proportio molaris guanini + cytosini in acido deoxyribonucleico: 36.6 mol %. Systema ubiquinoni: Q-9.

Holotypus: Separatus ex fuscosaccharo facto in Taiwania, 15. v. 1984, K. Tokuoka 29-25A, IAM 13112 conservatur in collectionibus culturarum quas 'Institute of Applied Microbiology, University of Tokyo,' Tokyo sustentat.

Growth in 25% (w/w) glucose- YM broth: After 3 days at 25°C, growth is very poor. After one week, the cells are globose to subglobose, 4.2-7.0 x 4.2-7.3 μm, and occur singly, in pairs, in short branched chains, or in clusters (Fig. I-2). After one month only a sediment is present.

Growth on 25% (w/w) glucose-YM agar: After one month at 25°C, the streak culture is cream-colored, dull, dry, smooth, and entire at the margin.

Slide culture on 25% (w/w) glucose- corn meal agar: No pseudomycelium is formed.

Sporulation: Ascospore, ballistospore, and teliospore are not formed.

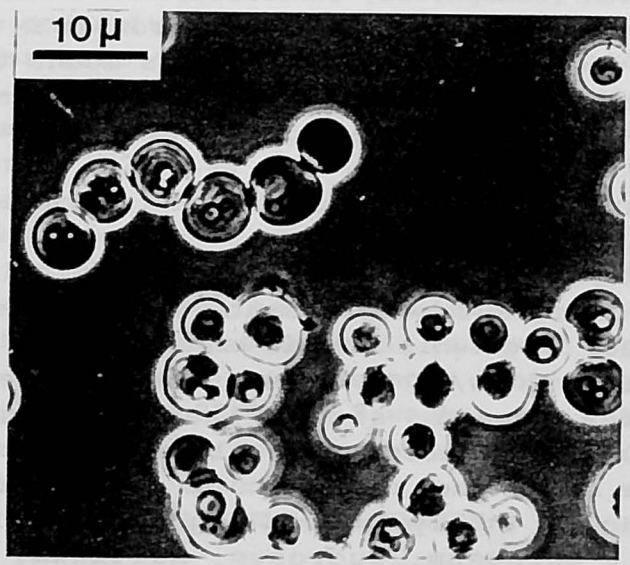


Fig. I-2 Vegetative cells of *Candida glucosophila* 29-25A. After one week at 25°C in 25% (w/w) glucose-YM broth.

Fermentation (in media containing 10% (w/v) NaCl): Only glucose is fermented.

Assimilation of carbon compounds (in media containing 10% (w/v) NaCl): Glucose, D-xylose (weak), and glycerol (weak) are assimilated, but not galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, L-arabinose, D-arabinose, D-ribose, L-rhamnose, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, or inositol.

Assimilation of potassium nitrate: Positive.

Assimilation of ethylamine HCl: Positive.

Assimilation of L-lysine: Negative.

Growth in vitamin-free medium: Negative.

Growth at 37°C: Positive.

Growth at 42°C: Negative.

No growth occurs on YM agar, the best growth is on 25% (w/w) glucose- YM agar.

Growth on 60% (w/w) glucose- YM agar: Positive.

Growth in the presence of 100 ppm of cycloheximide: Negative.

Hydrolysis of urea: Negative.

Production of extracellular DNase: Negative.

Color reaction with DBB: Negative.

G+C content of nuclear DNA: 36.6 mol%.

Ubiquinone system: Q-9.

Habitat: Brown sugar; Taiwan.

Strain examined: Isolated from brown sugar made in Taiwan, 15. v. 1984, K. Tokuoka 29-25A (IAM 13112; holotype).

Etymology: glu. co. so. phil. a; M. L. *glucosum* glucose; Gr. adj. *philus* loving; M. L. adj. *glucosophila* loving glucose, referring to the good growth in the presence of high concentrations of glucose.

The properties of the isolate 29-25A resembles those of *C. halonitratophila* (1) among osmophilic yeasts, but assimilation of D-xylose and D-mannitol, growth temperature, growth on YM agar, and G + C content of DNA do not fit. The isolate has 36.6 mol% guanine plus cytosine and *C. halonitratophila* IFO 1595 has 50.1 mol%, as determined in the present study. Based on these facts, the isolate 29-25A is identified as a new species in the genus *Candida*, and *Candida glucosophila* is proposed. This yeast is osmophilic (Table I-4).

2. *Candida dulciaminis* Tokuoka, Ishitani, Goto et Komagata sp. nov.

In liquido "YM", post dies 3 ad 25°C, cellulae ovoideae,

1.9-3.8 x 2.6-4.9 μm, singulae, binae, vel subinde in catenis brevis. In agaro "YM", post unum mensem ad 25°C, cultura cremea laevis, leviter mucosa, margine integra. Pseudomycelium nullum. Ascosporae nullae, ballistosporae nullae et teliosporae nullae. Fermentatio nulla. Glucosum, L-sorbosum, sucrosum, cellobiosum, trehalosum, lactosum, raffinosum, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, glycerolum, erythritolum, ribitolum, galactitolum (exiguum), D-mannitolum, D-glucitolum, salicinum et acidum succinicum assimilantur at non galactosum, maltosum, melibiosum, melezitosum, inulinum, amylum solbile, L-rhamnosum, alpha-methyl-D-glucosidum, acidum DL-lacticum, acidum citricum nec inositolum. Kalium nitricum non assimilatur. Leniter crescit sine vitaminis. Ureum hydrolysatur. Diazonium caeruleum B: positivum. Proportio molaris guanini + cytosini in acido deoxyribonucleico: 52.6 mol %. Systema ubiquinoni: Q-9.

Holotypus: Separatus ex dulciamine, Ibaraki Pref., Japonia, 20. v. 1984, K. Tokuoka 155-CA, IAM 13114 conservatur in collectionibus culturarum quas 'Institute of Applied Microbiology, University of Tokyo,' Tokyo sustentat.

Growth in YM broth: After 3 days at 25°C, the cells are ovoid, 1.9-3.8 x 2.6-4.9 μm, and occur singly, in pairs or occasionally in short chains (Fig. I-3). Only a sediment is formed.

Growth on YM agar: After one month at 25°C, the streak culture is cream-colored, smooth, slightly mucous, and flat with an entire border.

Slide culture on corn meal agar: No pseudomycelium is formed.

Sporulation: Ascospore, ballistospore, and teliospore are not formed.

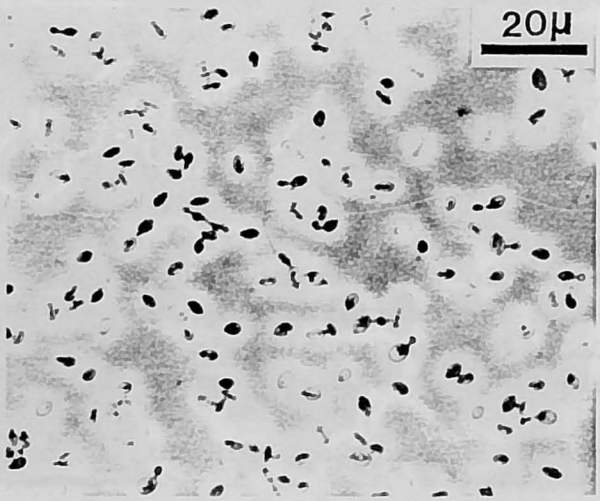


Fig. I-3 Vegetative cells of *Candida dulciaminis* 155-CA. After 3 days at 25°C in YM broth.

Fermentation: Absent.

Assimilation of carbon compounds: Glucose, L-sorbose, sucrose, cellobiose, trehalose, lactose, raffinose, D-xylose, L-arabinose, D-arabinose, D-ribose, glycerol, erythritol, ribitol, galactitol (weak), D-mannitol, D-glucitol, salicin, and succinic acid are assimilated, but not galactose, maltose, melibiose, melezitose, inulin, soluble starch, L-rhamnose, α-methyl-D-glucoside, DL-lactic acid, citric acid, or inositol.

Assimilation of potassium nitrate: Negative.

Growth in vitamin-free medium: Weak growth.

Growth at 30°C: Positive.

Growth at 37°C: Negative.

Growth on 60% (w/w) glucose- YM agar: Positive.

Hydrolysis of urea: Positive.

Production of extracellular DNase: Positive.

Color reaction with DBB: Positive.

G+C content of nuclear DNA: 52.6 mol%.

Ubiquinone system: Q-9.

Habitat: Sponge cake; Japan.

Strain examined: Isolated from sponge cake, Ibaraki Pref., Japan, 20. v. 1984, K. Tokuoka 155-CA (IAM 13114; holotype).

Etymology: dul. ci. a'min. is; L. gen. n. *dulciaminis* of a confectionery (sponge cake), referring to the source from which the yeast was isolated.

The present isolate belongs to a member of basidiomycetous yeasts because of positive activities of urease and extracellular DNase, positive color reaction with Diazonium Blue B, and higher G + C content of DNA. Although most of basidiomycetous yeasts can not grow at low $a_w$, this isolate grows even on 60% (w/w) glucose-YM agar ($a_w$ 0.826) as shown in Table I-4, and has significantly high sugar-tolerance. Since the isolate does not produce starch-like compounds and carotenoid pigment, and does not assimilate inositol, it is not classified in the genera *Cryptococcus* or *Rhodotorula*. Its physiological and biochemical properties, except for assimilation of citric acid and growth at 37°C, fit those of *Sterigmatomyces elviae* (1). However, it does not form sterigmata, which the cells of the genus *Sterigmatomyces* produce. According to "Yeasts"(20), the genus *Candida* is to be restricted to asexual yeasts with ascomycetous characteristics, while asexual yeasts with basidiomycetous characteristics will be accommodated in the genera *Cryptococcus* and *Rhodotorula*. However, the genus *Candida* in "The Yeasts" (1) and "Yeasts" (20) includes some species, such as *Candida antarctica*, *Candida aquatica*, and *Candida bacarum*, with positive urease activity, positive color reaction with Diazonium Blue B, and higher G+C content in DNAs. This shows the

inevitable accommodation in *Candida* of species not clearly fitting in the generic concepts of *Cryptococcus* and *Rhodotorula*. Following this, the present isolate is classified in the genus *Candida*. Since its properties do not fit the description of any known species, we propose a new species and name it *Candida dulciaminis*.

3. *Candida floricola* Tokuoka, Ishitani, Goto et Komagata sp. nov.

In liquido "YM", post dies 3 ad 25°C, cellulae subovoideae, 1.6-3.7 x 2.3-3.9 μm, singulae, binae, vel in catenis brevis. In agaro "YM", post unum mensem ad 25°C, cultura cremea, nitida, mollis, laevis, margine integra. Pseudomycelium nullum. Ascosporae nullae, ballistosporae nullae et teliosporae nullae. Glucosum, sucrosum (tardum), maltosum (tardum) et raffinosum (valde exiguum) fermentantur at non galactosum nac lactosum. Glucosum, galactosum, L-sorbosum, sucrosum, maltosum, raffinosum (valde exiguum), amylum solbile (exiguum), D-xylose (exiguum), D-ribosum, glycerolum, D-mannitolum, D-glucitolum et acidum succinicum assimilantur at non cellobiosum, trehalosum, lactosum, melibiosum, melezitosum, inulinum, L-arabinosum, D-arabinosum, L-rhamnosum, erythritolum, ribitolum, galactitolum, alpha-methyl-D-glucosidum, salicinum, acidum DL-lacticum, acidum citricum nec inositolum. Kalium nitricum non assimilatur. Ad crescentiam vitaminae externae necessariae sunt. Ureum non hydrolysatur. Diazonium caeruleum B: negativum. Proportio molaris guanini + cytosini in acido deoxyribonucleico: 51.7 mol %. Systema ubiquinoni: Q-9.

Holotypus: Separatus ex flore *Taraxaci platycarpi* in praedio Universitis Tokyoensis, Yayoi, Tokyo, Japonia, 15. v. 1984, K. Tokuoka 20-50A, IAM 13115 conservatur in collectionibus culturarum quas 'Institute of Applied Microbiology, University of Tokyo,' Tokyo sustentat.

Growth in YM broth: After 3 days at 25°C, the cells are short-ovoid 1.6-3.7 x 2.3-3.9 μm (Fig. I-4). They occur singly, in pairs, or in short chains. After one month a sediment and a very slight ring are present.

Growth on YM agar: After one month at 25°C, the streak culture is cream-colored, glossy, soft, smooth, and entire at the margin.

Slide culture on corn meal agar: No pseudomycelium is formed.

Sporulation: Ascospore, ballistospore, and teliospore are not formed.

Fermentation: Glucose, sucrose (slow), maltose (slow), and raffinose (very weak) are fermented, but galactose is not.

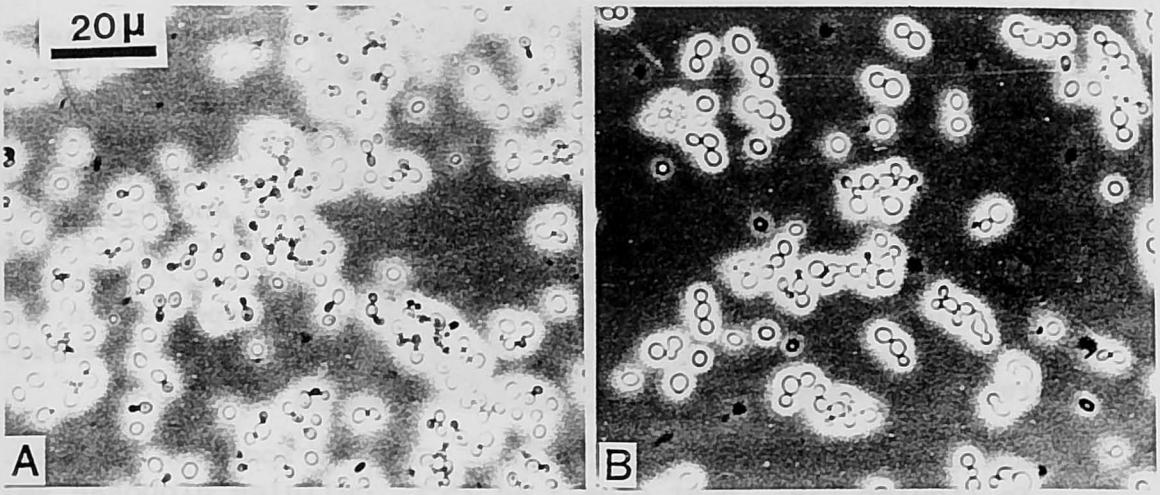


Fig. I-4 Vegetative cells of *Candida floricola* 20-50A and 34-25EA. After 3 days at 25°C in YM broth. A, Strain 20-50A. B, Strain 35-25EA.

Assimilation of carbon compounds: Glucose, galactose, L-sorbose, sucrose, maltose, raffinose (very weak), soluble starch (weak), D-xylose (weak), D-ribose, glycerol, D-mannitol, D-glucitol, and succinic acid are assimilated, but not cellobiose, trehalose, lactose, melibiose, melezitose, inulin, L-arabinose, D-arabinose, L-rhamnose, erythritol, ribitol, galactitol, α-methyl-D-glucoside, salicin, DL-lactic acid, citric acid, or inositol.

Assimilation of potassium nitrate: Negative.

Growth in vitamin-free medium: Negative.

Growth on 60% (w/w) glucose- YM agar: Positive.

Growth at 30°C: Positive.

Growth at 37°C: Negative.

Hydrolysis of urea: Negative.

Production of extracellular DNase: Negative.

Color reaction with DBB: Negative.

G+C content of nuclear DNA: 51.7 mol%.

Ubiquinone system: Q-9.

Habitat: Flowers of dandelion (*Taraxacum platycarpum* Dahlst.) and azalea (*Rhododendron* sp.); Japan.

Strain examined: Isolated from dandelion flowers (*Taraxacum platycarpum* Dahlst.) growing at the Farm of the University of Tokyo, Yayoi, Tokyo, Japan, 15. v. 1984, K. Tokuoka 20-50A (IAM 13115; holotype); isolated from azalea flowers (*Rhododendron* sp.) at the same locality, 15.v. 1984, K. Tokuoka 34-25EA (IAM 13116).

Etymology: flo. ri'co. la; L. n. *flos* flower; L. substantive ending *-cola* inhabitant; M. L. n. *floricola* inhabitant of

flowers.

Two isolates, 20-50A and 34-25EA, have the same morphological, physiological, and biochemical characteristics, and have 51.7 mol% guanine plus cytosine each in their DNAs. These strains resemble *Candida bombicola* (1) and *Candida etchellsii* (1). However, they differ from *C. bombicola* in fermentation of maltose, assimilation of maltose and D-ribose, and growth at 37°C, and they differ from *C. etchellsii* in fermentation of sucrose and assimilation of sucrose, D-xylose, and nitrate. The isolates are identified as a new species in the genus *Candida* and named *Candida floricola*. This species is sugar-tolerant (Table I-4).

4. *Candida vaccinii* Tokuoka, Ishitani, Goto et Komagata sp. nov.

In liquido "YM", post dies 3 ad 25°C, cellulae globosae vel subovoidae, 1.6-3.9 x 2.3-5.3 μm, singulae, binae, vel in catenis brevis. In agaro "YM", post unum mensem ad 25°C, cultura cremea, nitida, mollis, laevis, margine integra. Pseudomycelium nullum. Ascosporae nullae, ballistosporae nullae et teliosporae nullae. Glucosum, sucrosum (tardum) et raffinosum (valde exiguum) fermentantur at non galactosum, maltosum nec lactosum. Glucosum, galactosum (exiguum), L-sorbosum, sucrosum, cellobiosum, raffinosum, amylum solbile (exiguum), D-ribosum (exiguum), glycerolum, D-mannitolum, D-glucitolum, salicinum, acidum succinicum, et acidum citricum assimilantur at non maltosum, trehalosum, lactosum, melibiosum, melezitosum, inulinum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, erythritolum, ribitolum, galactitolum, alpha-methyl-D-glucosidum, acidum DL-lacticum, nec inositolum. Kalium nitricum assimilatur. Leniter crescit sine vitaminis. Ureum non hydrolysatur. Diazonium caeruleum B: negativum. Proportio molaris guanini + cytosini in acido deoxyribonucleico: 52.0 mol %. Systema ubiquinoni: Q-9.

Holotypus: Separatus ex flore *Vaccinii* in praedio Universitis Tokyoensis, Yayoi, Tokyo, Japonia, 15.v. 1984, K. Tokuoka 10-50A, IAM 13117 conservatur in collectionibus culturarum quas 'Institute of Applied Microbiology, University of Tokyo,' Tokyo sustentat.

Growth in YM broth: After 3 days at 25°C, cells are globose to short-ovoid, 1.6-3.9 x 2.3-5.3 μm, occur singly, in pairs, and in short chains and groups (Fig. I-5). After one month a sediment is present, but no pellicle and ring.

Growth on YM agar: After one month at 25°C, the streak culture is cream-colored, glossy, soft, smooth, and entire at the margin.

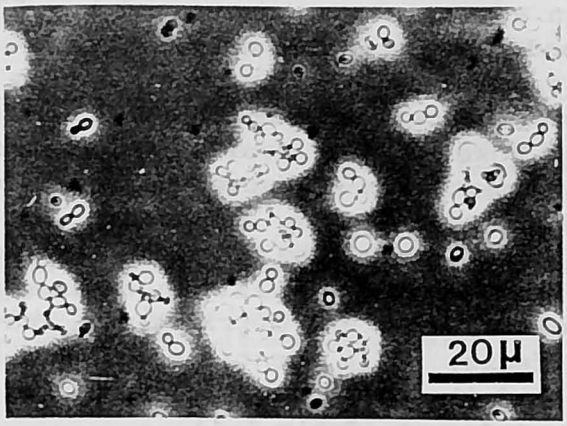


Fig. I-5 Vegetative cells of *Candida vaccinii* 10-50A. After 3 days at 25°C in YM broth.

Slide culture on corn meal agar: No pseudomycelium is formed.

Sporulation: Ascospore, ballistospore, and teliospore are not formed.

Fermentation: Glucose and sucrose (slow), and raffinose (very weak) are fermented, but not galactose, maltose, or lactose.

Assimilation of carbon compounds: Glucose, galactose (weak), L-sorbose, sucrose, cellobiose, raffinose, soluble starch (weak), D-ribose (weak), glycerol, D-mannitol, D-glucitol, salicin, succinic acid, and citric acid are assimilated, but not maltose, trehalose, lactose, melibiose, melezitose, inulin, D-xylose, L-arabinose, D-arabinose, L-rhamnose, erythritol, ribitol, galactitol, α-methyl-D-glucoside, DL-lactic acid, or inositol.

Assimilation of potassium nitrate: Positive.

Growth in vitamin-free medium: Weak growth.

Growth on 60% (w/w) glucose- YM agar: Positive.

Growth at 37°C: Positive.

Growth at 42°C: Negative.

Hydrolysis of urea: Negative.

Production of extracellular DNase: Negative.

Color reaction with DBB: Negative.

G+C content of nuclear DNA: 52.0 mol%.

Ubiquinone system: Q-9.

Habitat: Flower of blueberry (*Vaccinium* sp.); Japan.

Strain examined: Isolated from blueberry flowers (*Vaccinium* sp.) growing at the Farm of the University of Tokyo, Yayoi, Tokyo, Japan, 15.v.1984, K. Tokuoka 10-50A (IAM 13117; holotype).

Etymology: vac. cin'i.i; M. L. neut.n. *Vaccinium* generic name of the blueberry; M. L. gen. n. *vaccinii* of *Vaccinium*, referring to the host plant from which the yeast was isolated.

This strain assimilates nitrate and has 52.0 mol% guanine plus cytosine and a Q-9 ubiquinone system. These properties match those of the salt-tolerant *Candida* species useful for the production of soy sauce, such as *C. etchellsii* (1) and *Candida versatilis* (1). However, the isolate is distinguished from *C. etchellsii* by fermentation of sucrose and maltose, assimilation of sucrose, maltose, cellobiose, raffinose, and citric acid, and growth at 37°C, and differs from *C. versatilis* in fermentation of galactose and maltose, assimilation of L-sorbose, maltose, trehalose, succinic acid, and citric acid, and growth at 37°C. Based on these facts, it is identified as a new species in the genus *Candida*, and the name *Candida vaccinii* is proposed. This species is sugar-tolerant (Table I-4).

Judging from the results in I-1 and in this study, a considerable number of yeasts are considered to be osmophilic or sugar-tolerant, and they are isolated from foods containing relatively high concentrations of sugar and related materials. Further examination of the detailed characteristics of the osmophilic or sugar-tolerant yeasts is needed for taxonomy and for food preservation.

## I-3. *Sympodiomycopsis*: a new yeast-like anamorph genus with basidiomycetous nature from orchid nectar

During a survey of yeasts in high-sugar foods and related materials, an undescribed yeast-like fungus was isolated from orchid nectar (*Paphiopedilum primurinum*) collected at the Koishikawa Botanic Garden of the University of Tokyo, Tokyo in May 1985.

This fungus produces enteroblastic-annelloconidia and holoblastic-sympodioconidia which are neither ballistosporogenous nor sterigmatosporogenous, and has Q-10 as its major ubiquinone system and 56.3 mol% guanine plus cytosine (G + C) in its nuclear DNA. The fungus resembles *Sympodiomyces*, *Sporobolomyces*, *Sterigmatomyces* and related genera in one or two features at the generic level. However, these are not appropriate to accommodate the fungus. This paper introduces the new generic and species name *Sympodiomycopsis paphiopedili* to accommodate the fungus, for which descriptions and illustrations are provided below.

### MATERIALS AND METHODS

*Strains used.* The isolate and strains examined in this study

Table I-5 Strains examined.

| Species designation | Strain[1] | IAM no.[1] | Source |
|---|---|---|---|
| *Sympodiomycopsis paphiopedili* | 101-40A$^T$ (Isolate) | 13459$^T$ | Orchid nectar, Japan |
| *Sympodiomyces parvus* | CBS 6147$^T$ | 13472$^T$ | Antarctic sea water |
| *Sporidiobolus salmonicolor*[2] | YK 1026$^T$ | 13492$^T$ | Culture contaminant (mating type A1) |
| *Fellomyces polyborus* | CBS 6072$^T$ | 13471$^T$ | Tunnels of *Xyleborus torquatus* in *Cussonia umbellifera* |

[1]Abbreviations:$^T$, strain derived from the holotype; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; YK, Division of Microbial Taxonomy, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan (cf. Sugiyama et al. 1985).
[2]The anamorph is *Sporobolomyces salmonicolor* (Fischer et Brebeck) Kluyver et van Niel.

for comparison are listed in Table I-5.

*Phenotypic characterization.* The morphological and physiological properties of the isolate were investigated by the methods described by van der Walt and Yarrow (21). The urease test was performed on Christensen's urea agar (22), and results were recorded after five days of incubation at 25°C. The test for the extracellular deoxyribonuclease (DNase) activity was made by the method of Sen and Komagata (19). The Diazonium Blue B (DBB) color test was performed as described by van der Walt and Hopsu-Havu (23).

*Formation of carotenoid pigments.* Carotenoid pigments of the isolate, *Sympodiomyces parvus* CBS 6147$^T$, and *Fellomyces polybolus* CBS 6072$^T$ were determined by the method of Hasegawa et al. (24). *Rhodotorula rubra* (Demme) Lodder strain 70-25A described in I-1 was used for comparison.

*Chemotaxonomic characterization.* The cellular carbohydrate composition in the whole cell hydrolyzates for the isolate, *S. parvus* CBS 6147$^T$, *Sporidiobolus salmonicolor* YK 1026$^T$, and *Fellomyces polyborus* CBS 6026$^T$ was determined by using a Shimadzu GC-9A gas chromatograph equipped with a hydrogen flame ionization detector according to the method described by Sugiyama et al. (25). The DNA base composition and ubiquinone system of the isolate and *S. parvus* CBS 6147$^T$ were also determined as described in I-1.

*Ultrastructural studies on the cell wall, conidiogenesis and septal pores.* For transmission electron microscopy (TEM) cells were grown in YM broth in shake culture at 25°C for 48 hr. The centrifuged, washed cells were fixed with 3% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) for 30 min at room temperature and then postfixed for 1 hr with 2% aqueous $KMnO_4$, also at room temperature. In either cases, the cells were rinsed with water. After pre-staining with 0.5% uranyl acetate for 2 hr, the cells were embedded in agar at 45°C and diced into 1-2 mm square

pieces. These were dehydrated through increasing concentrations of ethanol and absolute acetone and finally embedded in Spurr's resin (26); Quetol 653 was used instead of Spurr's D.E.R. 736. Ultrathin sections were cut with a diamond knife, poststained with Reynolds lead citrate (27) for 10 min and examined a JEOL 200 CX electron microscope at 100kV. Terms for the mode of conidiogenesis (conidium ontogeny) were based on those employed by Hawksworth et al. (28).

## RESULTS

The production of carotenoid pigments, the cellular carbohydrate composition, the ubiquinone systems, and the DNA base composition in *Sympodiomycopsis paphiopedili* gen. nov. et sp. nov., *Sympodiomyces parvus*, *Sporidiobolus salmonicolor* Fell et Statzell Tallman (anamorph: *Sporobolomyces salmonicolor* (Fischer et Brebeck) Kluyver et van Niel), and *Fellomyces polyborus* are summarized in Table I-6 with previously reported data for the carotenoid pigments (29), ubiquinone systems (30, 31), and DNA base composition (32, 33).

*Carotenoid pigments*

The absorption spectra of pigments from *Rhodotorula rubra* strain 70-25A agreed with those of carotenoid pigments; i.e., its absorption maximum was 480 nm with one shoulder at 460 nm and another shoulder at 510 nm. On the other hand, *Sympodiomycopsis paphiopedili* 101-40A$^T$, *Sympodiomyces parvus* CBS 6147$^T$, and *Fellomyces polyborus* CBS 6072$^T$ did not show any absorption between 350 to 550 nm. Therefore, carotenoid pigments were absent in these strains.

*Cellular carbohydrate composition*

*Sympodiomycopsis paphiopedili* 101-40A$^T$ contained a trace amount (less than 0.1 mol%) of xylose in the cells at a sensitivity 2.5 times higher to the standard (Table I-6).

*Sympodiomyces parvus* CBS 6147$^T$ and *Sporidiobolus salmonicolor* YK 1026$^T$ lacked xylose in the cells, whereas *Fellomyces polyborus* CBS 6072$^T$ showed the presence of xylose in the cells. Mannose and glucose, or glucose was detected as the predominant sugar(s) in all strains tested.

*Sympodiomycopsis paphiopedili* 101-40A$^T$ contained relatively high amount of galactose in the cells, whereas *Sympodiomyces parvus* CBS 6147$^T$, *Sporidiobolus salmonicolor* YK 1026$^T$ and *Fellomyces polyborus* CBS 6072$^T$ contained low amounts of galactose in the cells. The remaining sugars, i.e., rhamnose, fucose,

Table I-6 Carotenoid pigments, cellular carbohydrate composition, ubiquinone systems, and DNA base composition of examined strains.*

| | *Sympodiomycopsis paphiopedili* 101–40A$^T$ | *Sympodiomyces parvus* CBS 6147$^T$ | *Sporidiobolus salmonicolor* YK 1026$^T$ | *Fellomyces polyborus* CBS 6072$^T$ |
|---|---|---|---|---|
| Carotenoid pigments | – | – | +[1] | – |
| Cellular carbohydrate composition (mol%) | | | | |
| rhamnose | – | – | – | – |
| fucose | – | – | 4.2 | – |
| ribose | 2.2 | 2.4 | 1.5 | 1.9 |
| arabinose | 0.5 | 1.2 | 0.6 | 2.5 |
| xylose | tr | – | – | 2.6 |
| mannose | 5.5 | 45.9 | 40.6 | 14.3 |
| glucose | 79.2 | 42.4 | 45.9 | 76.0 |
| galactose | 12.6 | 8.1 | 7.2 | 2.7 |
| Major ubiquinone system | Q-10 | Q-9 | Q-10[2] | Q-10[4] |
| Mol% G + C | 56.3 | 46.3 | 63.5[3] | 49.6[5] |

* Abbreviations: +, present; –, absent; tr, trace amount of a sugar.
[1] From Fell & Statzell Tallman (1984a)
[2] From Nakase & Suzuki (1986)
[3] From Storck et al. (1969)
[4] From Yamada & Banno (1984a)
[5] From Fell et al. (1984)

ribose, and arabinose, were absent or only present in small amounts.

*Ubiquinone systems and DNA base composition*

*Sympodiomycopsis paphiopedilli* 101-40A$^T$ had the major ubiquinone system Q-10 and 56.3 mol% G + C, whereas *Sympodiomyces parvus* CBS 6147$^T$ had the major ubiquinone system Q-9 and 46.3 G+C mol% (Table I-6).

*Ultrastructural studies on the cell wall, conidiogenesis and septal pores*

TEM micrographs show that the cell wall of *S. paphiopedilli* is composed of multilayers (Fig. I-8 c, d). The cell wall structure observed agrees well with that of Kreger-van Rij and Veenhuis (34) reported for typical basidiomycetous yeast taxa. The mode of conidiogenesis of yeast and elongated cells are very complicated. In the early stage yeast cell phase of that is enteroblastic-annellidic, and each conidium is formed as a bud from an inner layer of mother cell (Fig. I-8 a-c). But after one or two times of annellidic proliferation, the conidiogenous tip is elongated (elongated cell phase) and changed to be holoblastic-sympodial as showing in Fig. I-8 e-f (cf. Fig. I-6 f). A conidium is formed at one side of elongated conidiogenous tip and successively another conidiogenesis is occurred at the newly developed apex. The conidiogenesis of sympodial stage at elongated conidiogenous cells seems to be holoblastic.

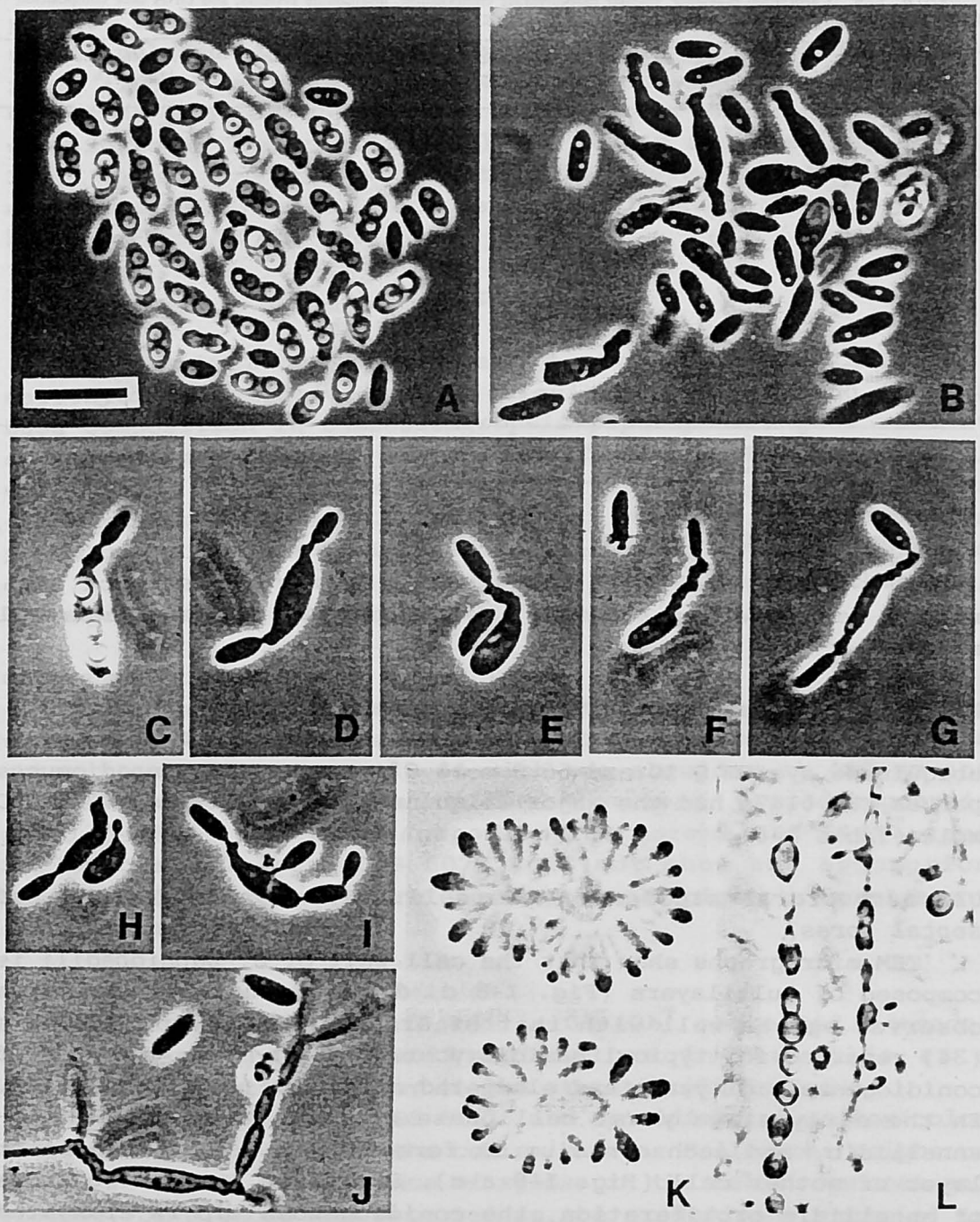


Fig. I-6. *Sympodiomycopsis paphiopedili* 101-40A$^T$ in malt extract after 4 to 7 days (A-J, L) and after 12 days (K) at 25°C. (A) Vegetative yeast cells. (B-I) Vegetative showing sympodial, geniculate, denticulate growth, some of them bearing successive terminal conidia. (J) A cell forming a pseudomycelium. (K) Two vegetative yeast cells showing multilateral (sympodial) budding. (L) Torulose hyphae in a pellicle on which holoblastic, sessile conidia are directly formed. Bar = 10 μm.

Septal pore ultrastructures of *S. paphiopedili* resemble those found in members of the Ustilaginales (35). Their TEM micrographs obtained are not included here and will be presented elsewhere.

Description

*Sympodiomycopsis* Sugiyama, Tokuoka et Komagata, gen. nov. Genus ad Deuteromycotina, Hyphomycetes pertinens.

Coloniae in agaro malti cremeae, sordidae, laeves, molles, leviter mucosae. Conidiophora indistincta. Cellulae zymoticae praesentes; gemmatio praecipur enteroblastico-annellidica vel raro holoblastico-sympodialis. Mycelium verum interdum evolutum. Cellula conidiogena ex cellulis zymotica aut ex hyphis indistinctis oriunda. Cellulae conidiogenae polyblasticae, terminales, intercalares aut sparsae, cylindricae, saepe geniculatae, denticulatae; 3 vel pluria conidia holoblastica in successione sympodiali in parte apicali formantia. Conidia solitaria, acropleurogena, obclavata ad obovoidea, continua, hyalina, laevia, tenuitunicata, basi acuminata. Ascosporae, teliosporae, ballistosporae vel sterigmatosporae nullae. Fermentatio nulla. Inositolum assimilatur. Kalium nitricum assimilatur. Materia amyloidea iodophila non formatur. Ureum hydrolysatur. Acidum deoxyribonucleinicum finditur. Diazonium caeruleum B negativum. Pigmenta carotenoidea non formantur. Xylosum in cellulis praesens in quantite rudimentali. Systema ubiquinoni majus; Q-10.

Species typica: *Sympodiomycopsis paphiopedili* Sugiyama, Tokuoka et Komagata.

Colonies on malt agar are dull cream-colored; the surface is smooth; the texture is soft and slightly mucous. Yeast cells are present; the budding is principally holoblastic-annellidic and rarely holoblastic-sympodial. True mycelium is sometimes developed. Conidiophores are micronematous, mononematous, usually and unbranched (but sometimes branched). Conidiogenous cells develop directly from a yeast cell or from undifferentiated hyphae. Conidiogenous cells are polyblastic, terminal, intercalary or scattered, cylindrical, often geniculate, denticulate; the apical part forms 3 to several holoblastic conidia in sympodial order and a thin cluster of conidium-bearing denticles which may form a short geniculate rachis; denticles conspicuous. Conidia are solitary, acropleurogenous, obclavate or obovoid, 1-celled, hyaline, smooth, thin-walled, with acuminate

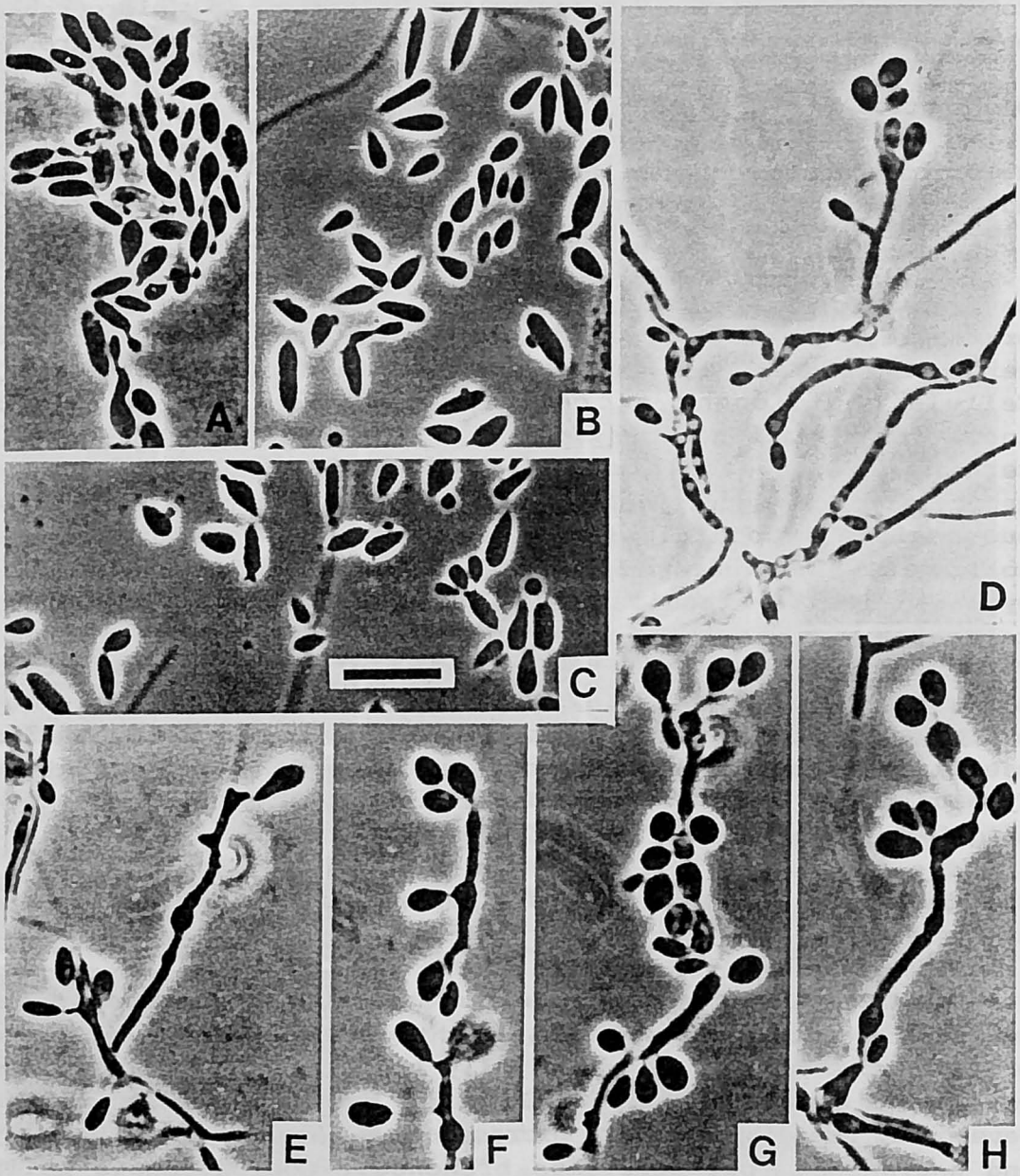


Fig.I-7. *Sympodiomycopsis paphiopedili* 101-40A$^T$ on corn meal agar after 4 to 25 days at 25°C. (A) Vegetative yeast cells, some having a conidium after 4 days. (B,C)Vegetative yeast cells, some of them elongating after 2 weeks. (D-H) Undifferentiated hyphae having terminal, intercalary or scattered conidiogenous cells, some of them 3 to several conidia in sympodial order. Bar = 10 μm.

bases. Ascospores, teliospores, ballistospores, and sterigmatospores have not been observed.

No fermentation is observed. Inositol is assimilated. Nitrate is assimilated. Starch-like compounds are not formed. Urease test is positive. Extracellular DNase test is positive. Diazonium Blue B color test is positive. Carotenoid pigments are not produced. Xylose in the whole cell hydrolyzates is present in a trace amount. Major ubiquinone system is Q-10.

Type species: *Sympodiomycopsis paphiopedili* Sugiyama, Tokuoka et Komagata

*Etymology*

The generic name *Sympodiomycopsis* (Lat.n.fem.) was chosen because of having the sympodial conidiogenous cells, which are very similar to those found in *Sympodiomyces*.

*Sympodiomycopsis paphiopedili* Sugiyama, Tokuoka et Komagata, anam. -sp. nov. (Figs I-6 - I-8).

In liquido malti post 3 dies ad 25°C, cellulae ovoideae ad elongatae, interdum subglobosae, 4-11 x 2-6 μm, singulae, binae aut in catenis brevis, reproductione gemmiferam praecipue holoblastico-annelidicam, raro holoblastico-sympodialem. Cellula conidiogena ex cellulis zymotica oriunda et elongata ad 7 μm longa cicatricibus. Cellulae conidiogenae polyblasticae, indeterminatae, terminales, sympodiales, cylindricae, geniculatae, denticulatae. Conidia solitaria, acropleurogena, 1-celled, laevia, tenuitunicata, obclavata ad obovoidea, basi acuminata, 3-5 μm longa, ad partem latissima 1.5-2 μm crassa. In post 7 dies pellicula incrassata interdum formatur; pellicula ex mycelio vero cum cellulis conidiogenis indistinctis et cellulis zymoticis composita. In agaro malti post 3 dies ad 25°C, coloniae convexae, color cremeus et sordidus, pagina laevis, textura mollis, margo integer. In agaro farina *Zeae maydis* post 14 dies ad 25°C, mycelium verum interdum evolutum. Mycelium e hyphis hyalinis, laevibus, tenuitunicatis, septatis, ramosis, 1-2 μm crassis, interdum torulosis usque ad 5 μm crassis compositum. Conidiophora micronemata, mononemata, recta aut flexuosa, plerumque eramosa sed laxe ramosa, hyalina, laevia. Cellulae conidiogenae polyblasticae, indeterminatae, terminales et intercalares vel sparsae, cylindricae, saepe geniculatae, denticulatae; 3 vel pluria conidia holoblastica in successione sympodiali in parte apicali formatia; denticuli subulati, usque ad 3 μm longi. Conidia solitaria, acropleurogena, hyalina,

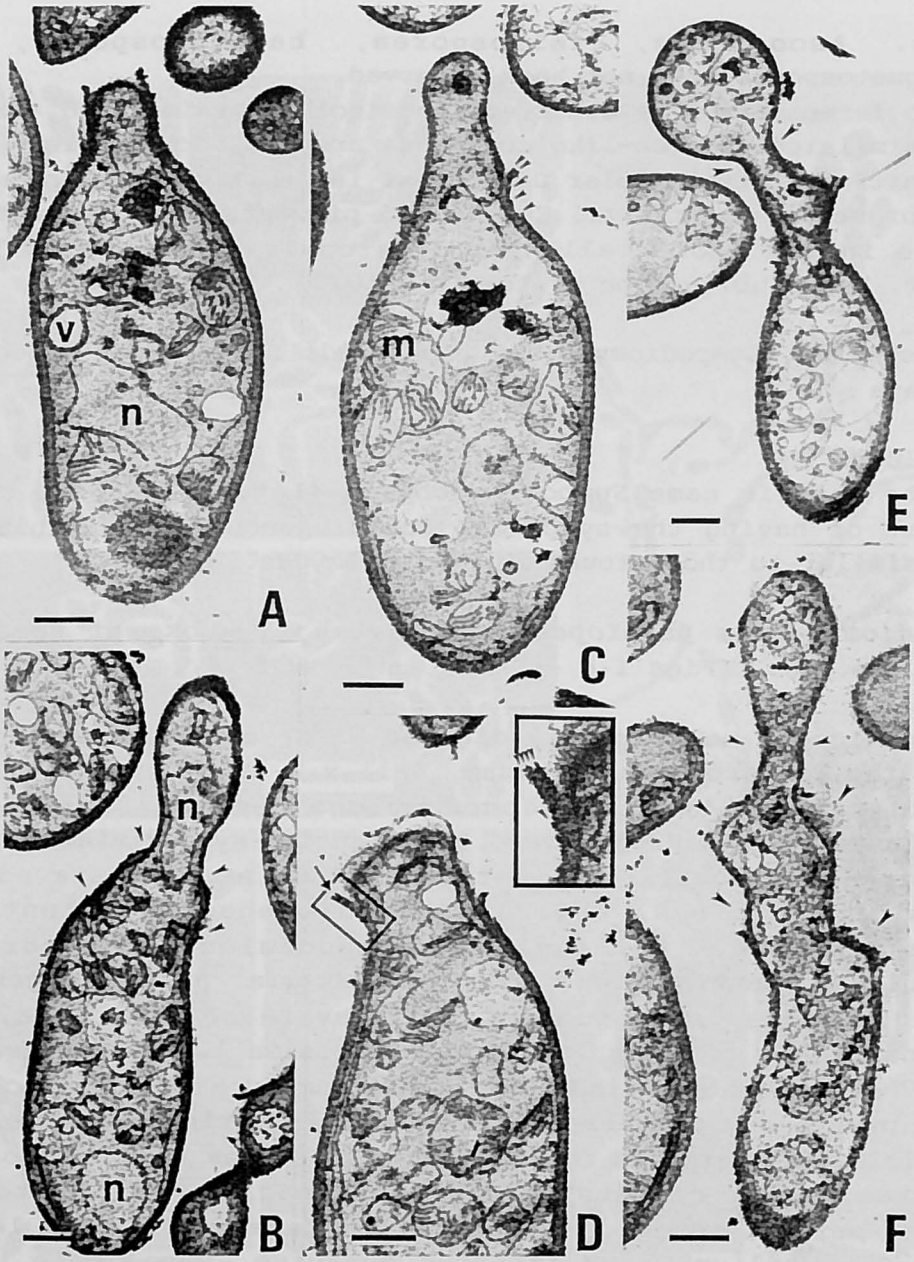


Fig. I-8. TEM micrographs of *Sympodiomycopsis paphiopedili* 101-40A$^T$. (A,B) Enteroblastic-annelidic conidiogenesis in young yeast cells. The wall of conidium is continuous with the inner layer of the mother cells (arrows). (C) The tip of conidiogenous cell is elongated and forms conidia. (D) After budding at the right side of the conidiogenous tip. Arrows of C and D indicate multilayered cell wall of the mother cell (in the box, x40,000). (E,F) Holoblastic-sympodial conidiogenesis in elongated cells. (E) A conidium was formed on another side of the tip, and the wall of conidium with continuous with that of the tip (arrows). (F) Successively another conidiogenesis is occured at the newly developed apex. Bar indicate 0.5 μm.

laevia, tenuitunicata, continua, obclavata ad obovoidea, basi acuminata, 3-6 µm longa, ad partem latissima 2-3 µm crassa. Ascosporae, teliosporae, ballistosporae et sterigmatosporae nullae. Fermentatio nulla. Glucosum, galactosum (valde exigue), L-sorbosum, sucrosum, maltosum, cellobiosum, trehalosum, lactosum (exigue), melibiosum, raffinosum, melezitosum, inulinum (exigue), amylum solubile (exigue), D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, glycerolum, erythritolum, D-mannitolum, D-glucitolum, α-methyl-D-glucosidum, DL-acidum lacticum (exigue) et inositolum assimilantur at non rhamnosum, ribitolum, galactitolum, salicinum nec acidum citricum. Kalium nitricum assimilatur. Ad crescentiam vitamina externa non necessaria sunt. Materia amyloidea iodophila non formatur. Ureum hydrolysatur. DNaseum positivum. Diazonium caeruleum B positivum. Pigmenta carotinoidea non formantur. Xylosum in cellulis praesens in quantite rudimentali. Systema ubiquinoni majus Q-10 adest. Proportio molaris guanini + cytosini in acido deoxyribonucleinico: 56.3 mol%. Teleomorphosis ignota.

*Holotypus*

Separatus ex nectare *Paphiopedili primurini*, 'Koishikawa Botanic Garden of the University of Tokyo, Bunkyo-ku, Tokyo,' 'Japonia, 24. v. 1985, leg. K. Tokuoka #101-40A, IAM-F-0145 (laminae); cultura IAM 13459 ex typo conservatur in collectionibus culturarum quas 'Institute of Applied Microbiology, The University of Tokyo, Tokyo' sustentat.

*Growth in malt extract*

After 3 days at 25°C the single cells vary from ovoid to elongate, sometimes subglobose, 4-11 x 2-6 µm, occurring singly, in pairs or in short chains, reproducing principally by enteroblastic-annellidic or rarely by holoblastic-sympodial budding. A conidiogenous cell develops directly from a yeast cell and elongates to 3.5 µm long with one to several scars; after 7 days an elongated conidiogenous cell, up to 7 µm long, is formed which forms successive holoblastic conidia. Conidiogenous cells are polyblastic, integrated, terminal, sympodial, cylindrical, sometimes geniculate, denticulate. Conidia are solitary, acropleurogenous, simple, 1-celled, hyaline, smooth, thin-walled, obclavate to obovoid, with acuminate bases, 3-5 µm long and 1.5-2 µm thick in the broadest part. After 7 days a thick pellicle is sometimes formed; it is composed of true mycelium with undifferentiated conidiogenous cells bearing blastoconidia and yeast cells; the hyphae are hyaline, smooth, thin-walled, septate, branched, 1.0-2.5 µm thick, often torulose and then up

Table I-7 Characteristics of *Sympodiomycopsis* and its similar genera.

| Generic name | *Sympodiomycopsis* Sugiyama et al.[1] | *Sympodiomyces* Fell et Statzell (1971)[2] | *Sporobolomyces* Kluyver et v. Niel (1925)[3] | *Bensingtonia* Ingold (1986) em. Nakase et Boekhout (1988)[4] | *Bullera* Derx (1930)[5] | *Ballisto-sporomyces* Nakase et al. (1989)[6] | *Sterigmatomyces* Fell (1966) em. Yamada et Banno (1984)[7] | *Tsuchiyaea* Yamada et al. (1988)[8] | *Fellomyces* Yamada et Banno (1984)[9] | *Kurtz-manomyces* Yamada et al. (1988)[10] |
|---|---|---|---|---|---|---|---|---|---|---|
| Type species | *S. paphiopedili* Sugiyama et al. | *S. parvus* Fell et Statzell | *S. roseus* Kluyver et v. Niel | *B. ciliata* Ingold | *B. alba* (Hanna) Derx | *B. xanthus* Nakase et al. | *S. halophilus* Fell | *T. wingfieldii* (van der Walt et al.) Yamada et al. | *F. polyborus* (Scott et v.d. Walt) Yamada et Banno | *K. nectairei* (Rodrigues de Miranda) Yamada et al. |
| Carotenoid pigments | − | − | + | v | v | + | v | − | v | + |
| True mycelium | + | + | v | v | − | v | − | − | v | − |
| Pseudomycelium | − | − | v | v | v | v | − | − | − | − |
| Ballistospores | − | − | + | + | + | + | − | − | − | − |
| Sterigmatospores | − | − | − | − | − | − | + | + | + | + |
| Fermentation | − | − | − | − | − | − | − | − | − | − |
| Assimilation of inositol | + | + | v | − | v | − | − | + | v | − |
| Assimilation of nitrate | + | − | v | v | v | v | v | − | v | + |
| Starch formation | − | − | v | − | + | − | − | w | v | − |
| Urease test | + | − | + | + | + | v | + | + | + | + |
| DNase test | + | + | + | NT | NT | NT | + | NT | + | NT |
| DBB color test | + | − | + | + | + | + | + | + | + | + |
| Xylose in the whole cell hydrolyzates | tr | − | − | − | + | − | − | + | + | − |
| Major ubiquinone system | 10 | 9 | 10,10($H_2$)[11] | 9 | 10 | 10 | 9 | 9 | 10 | 10 |
| Mol% G + C | 56.3 | 46.3 | 51.5–65.0 | 46.5–60.0 | 48.8–62.7 | 48.3–56.8 | 51.5–55.4 | 55.4 | 48.9–55.2 | 52.5 |
| Teleomorph | unknown | unknown | *Sporidiobolus* | unknown | unknown | unknown | unknown | | *Sterigmato-sporidium* | unknown |
| Phylogeny | bas | asc | bas | bas | bas | bas | bas | bas | bas | bas |
| Habitat | orchid nectar | sea water | various substrates | dead leaf | various substrates | dead leaf | various substrates | frass of a Scolytid beetle | various substrates | cheese |

* Abbreviations: +, present or positive reaction; −, absent or negative reaction; tr, trace amount of a sugar; v, variable; asc, ascomycetous affinity; bas, basidiomycetous affinity.

[1] From the present study.

[2] From Fell & Statzell 1971; Barnett et al. 1983; Kreger-van Rij 1984; Kocková-Kratochvílová 1982; van der Walt et al. 1986; Sen & Komagata 1979.

[3] From Barnett et al. 1983; Kreger-van Rij 1984; Fell & Statzell Tallman 1984a, b; Shivas & Rodrigues de Miranda 1983; Phaff & do Carmo-Sousa 1962; van der Walt et al. 1986; Nakase & Suzuki 1985, 1986c, 1987b, d; Sen & Komagata 1979; Hagler & Ahearn 1981; van der Walt & Hopsu-Havu 1976; von Arx & Weijman 1979; Weijman et al. 1982; Weijman & Rodrigues de Miranda 1983; Yamada & Kondo 1972, 1973; Nakase & Komagata 1968, 1971b; Storck et al. 1969; Storck & Alexopoulos 1970; Yarrow & Fell 1980; Suzuki & Nakase 1988; Nakase et al. 1987a; Nakase & Ito 1988.

[4] From Ingold 1986; Nakase & Boekhout 1988; Nakase et al. 1989a; Boekhout 1987; Nakase & Suzuki 1985, 1986b, 1987a-c, 1988; van der Walt et al. 1989; Suzuki & Nakase 1988; Nakase et al. 1987b; Yamada et al. 1988c.

[5] From van der Walt & Scott 1970; Barnett et al. 1983; Rodrigues de Miranda 1984; Nakase & Suzuki 1985, 1986a, c, d, 1987e; Nakase & Komagata 1971a, b; Suzuki & Nakase 1988; Buhagiar et al. 1983; Yamada et al. 1983; Weijman & Rodrigues de Miranda 1983; Komagata & Nakase 1965.

[6] From Nakase et al. 1989b.

[7] From Fell 1966; Soneck & Yarrow 1969; Barnett et al. 1983; Fell et al. 1984; Yamada & Banno 1984a, b; van der Walt et al. 1987; Hagler & Ahearn 1981; van der Walt & Hopsu-Havu 1976; Sugiyama et al. 1985; Nakase & Komagata 1971b; Yamada et al. 1983, 1988a.

[8] From Yamada et al. 1988b; van der Walt et al. 1987.

[9] From Scott & van der Walt 1970; Yamada & Banno 1984a, b; Barnett et al. 1983; Fell et al. 1984; Hagler & Ahearn 1981; van der Walt & Hopsu-Havu 1976; Kraepelin & Schulze 1982; Yamada et al. 1986, 1988a; Rodrigues de Miranda 1975.

[10] From Yamada et al. 1988a; Rodrigues de Miranda 1975; Kurtzman 1990.

[11] All *Sporobolomyces* spp. had the Q-10 system with the marked exception of *S. elongatus* having the Q-10($H_2$) system (Nakase & Suzuki 1986d).

to 5 μm thick. After 14 days, in addition to pseudohyphal cells in branched chains, cells with numerous ovoid blastoconidia, which are formed multilaterally are sometimes seen.

*Growth on malt agar*

After 3 days at 25°C the streak culture is dull, cream-colored; the surface is smooth; the texture is soft and slightly mucous.

*Slide culture on corn meal agar*

After 14 days at 25°C true mycelium is sometimes formed. Mycelium is composed of hyphae which are hyaline, smooth, thin-walled, septate, branched, 1-2 μm thick, often torulose and then up to 5 μm thick. Conidiophores are micronematous, mononematous, straight or flexuous, usually unbranched but sometimes loosely branched, hyaline, smooth. Conidiogenous cells are polyblastic, indeterminate, terminal, intercalarly or sparse, cylindrical, sometimes geniculate, denticulate; the apical part forms 3 to several holoblastic blastoconidia in sympodial order and a thin cluster of conidium-bearing denticles which may form a short rachis; denticles subulate, up to 3 μm long. Conidia are solitary, acropleurogenous, simple, 1-celled, hyaline, smooth, thin-walled, obclavate to obovoid, with acuminate bases, 3-6 μm long and 2-3 μm thick in the broadest part. Ascospores, teliospores, ballistospores and sterigmatospores have not been observed.

*Etymology*

The specific epithet *paphiopedili* (Lat. n. gen.) refers to the source of the isolate, i. e., the orchid genus *Paphiopedilum*.

*Material examined*

Isolated from nectar of *Paphiopedilum primurinum*, Koishikawa Botanic Garden of the University of Tokyo, Bunkyo-ku, Tokyo, Japan, 24. v. 1985, K. Tokuoka # 101-40A, IAM-F-0145 (slides; HOLOTYPE); the culture derived from the holotype has been deposited at the Culture Collection of the Institute of Applied Microbiology, The University of Tokyo, Tokyo, as IAM 13459.

## DISCUSSION

The present isolate (101-40A) was usually dominant as a yeast morph, although it was associated with a hyphal morph. The latter morph can easily be lost. In addition to the characteristic behavior, no teleomorph was found. Accordingly its taxonomic

position was seeked out among anamorphic yeasts.

The genus *Sympodiomycopsis* newly described here to accommodate the single isolate is characterized by no formation of carotenoid pigments, no formation of both ballistospores and sterigmatospores, a yeast morph with enteroblastic-annellidic (rarely holoblastic-sympodial) budding cells and a hyphal morph with holoblastic-sympodioconidia, lack of fermentative ability, ability to assimilate inositol and nitrate, inability to form starch-like compounds, positive reaction for urease, DNase and DBB color tests, presence of a trace amount of xylose in the whole cell hydrolyzates, and Q-10 ubiquinone system.

The distinction of *Sympodiomycopsis* from similar yeast and yeast-like anamorph genera is summarized in Table I-7. Differential characters among the ten genera are: formation of carotenoid pigments (negative for *Sympodiomycopsis*, *Sympodiomyces*, and *Tsuchiyaea*), formation of ballistospores (positive for *Sporobolomyces*, *Bensingtonia*, *Bullera*, and *Ballistosporomyces*), formation of sterigmatospores (positive for *Ballistosporomyces*, *Sterigmatomyces*, *Tsuchiyaea*, *Fellomyces*, and *Kurtzmanomyces*), the urease test (positive for the nine genera except *Sympodiomyces*), the DBB color test (positive for the nine genera except *Sympodiomyces*), xylose in the whole cell hydrolyzates (present for *Tsuchiyaea* and *Fellomyces*; and also present in a trace amount for *Sympodiomycopsis*), and the ubiquinone systems (Q-10 for *Sympodiomycopsis*, *Sporobolomyces*, *Bullera*, *Ballistosporomyces*, *Fellomyces*, and *Kurtzmanomyces*; Q-9 for *Sympodiomyces*, *Sterigmatomyces*, and *Tsuchiyaea*; and exceptionally Q-10($H_2$) for *Sporobolomyces elongatus*). Therefore, the ten genera are differentiated from each other by a specific combination of these characters.

Although the reason could not be defined, the datum on the cellular carbohydrate composition for *Sporidiobolus salmonicolor* YK 1026$^T$ was inconsistent with those previously reported by Sugiyama et al. (25) that *Sporidiobolus salmonicolor* YK 1026$^T$ (teleomorph of *Sporobolomyces salmonicolor*) had xylose in the cells. We would pay regard to the present study and conclude that *Sporidiobolus salmonicolor* YK 1026$^T$ lacks xylose in the cells. This determination has been supported by some researchers (36-38).

As mentioned above, the conidia of *Sympodiomycopsis paphiopedili* are neither ballistosporogenous nor sterigmatosporogenous. Therefore, this species is distinguished from all accepted species of the genera *Sporobolomyces* (20, 39-45), *Bensingtonia* (42, 43, 46-55), *Bullera* (20, 42, 56-61), *Sterigmatomyces* (20, 62-64), *Ballistosporomyces* (65), *Tsuchiyaea*

(66), *Fellomyces* (67), and *Kurtzmanomyces* (68). Morphologically *Sympodiomycopsis paphiopedilli* is very similar to *Sympodiomyces parvus* (20, 69, 70), which is characterized by a yeast morph and an inconspicuous hyphal morph. The mode of conidiogenesis is similar between these two genera. However, the true mycelium of *Sympodiomycopsis paphiopedilli* is more differentiate than that of *Sympodiomyces parvus*. The two genera are distinctly distinguished by their biochemical and chemotaxonomic characteristics: i. e., assimilation of nitrate, urease test, DBB color test, major ubiquinone system, and DNA base composition (see Table I-7).

*Sympodiomyces parvus* is considered to be an ascomycetous yeast by van der Walt and Hopsu-Havu (23) and Fell (70) because of negative urease and DBB color reactions, and a cell wall ultrastructure, which is typical of ascomycetous yeasts. In addition to these characters, a lower G + C content (46.3 mol%) obtained in this study further reinforces van der Walt and Hopsu-Havu's and Fell's supposition. We consider that *Sympodiomycopsis paphiopedilli* has a basidiomycetous affinity because of the following characters: the positive reaction for urease, DNase and DBB color tests, the Q-10 ubiquinone system, the relative high G + C content (56.3 mol%), and the basidiomycetous type of cell wall and septal pore ultrastructures found in members of the Ustilaginales. In addition to these characters, the presence of a trace amount of xylose in the cells reinforces this supposition.

In summary, *Sympodiomycopsis*, which is based on the type species *S. paphiopedilli*, is a non-carotenoid producing, non-fermentative, annellidically or sympodially reproducing yeast-like genus; the cell wall and septal pore ultrastructures and some chemotaxonomic characters indicate a basidiomycetous affinity.

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## CHAPTER II

## MINIMUM WATER ACTIVITIES FOR THE GROWTH OF THE ISOLATES

Water activity ($a_w$) is one of the important environmental factors affecting microbial growth and the minimum $a_w$ for growth are useful indicators in food preservation. In previous studies (1-4), many yeasts grew at $a_w$ higher than 0.88, while osmophilic or osmotolerant yeasts grew at lower $a_w$ levels. Some strains of the genus *Zygosaccharomyces*, typical osmophilic yeasts, were reported to tolerate an $a_w$ as low as 0.65 (5, 6), and the lowest minimum $a_w$ for growth recorded at present is 0.62 (7). Although the water relations of the genus *Zygosaccharomyces* were previously studied, there are few papers describing the minimum $a_w$ for the growth of sugar-tolerant yeasts, which belong to many genera and species as described in CHAPTER I.

In this study, the minimum $a_w$ for the growth of the isolates described in CHAPTER I were determined using four kinds of solutes. It has been found that they depend on the solutes for controlling $a_w$ and other environmental factors as well as yeast species and strains.

### MATERIALS AND METHODS

*Strains*. Among the isolates from a variety of high-sugar foods and related materials described in CHAPTER I, 35 yeast strains belonging to 31 species of 12 genera were tested. Five of those strains belonged to *Z. rouxii*, a representative osmophilic yeast. All the strains are stored in National Food Research Institute.

*Preparation of media and measurement of* $a_w$. YM agar slant and two kinds of agar slants containing 50% (w/w) glucose or 40% (w/w) fructose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract were prepared for preincubation. Three ml of broths containing 1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and different percentages of solutes for controlling $a_w$ ($a_w$-controlling solutes) were prepared in 7-ml test tubes. The test tubes were tightly sealed with screw-caps to prevent changes in the $a_w$ of the broths. Glucose, fructose, sucrose, and sodium chloride were used as $a_w$-controlling solutes. Three other kinds of broth were also used to prepare media rich in sodium chloride; Medium A: 90 ml of YM broth and 10 ml of miso extract, Medium B: 90 ml of koji extract, 10 ml of raw soy sauce, and 0.5% glucose, Medium C: 5% glucose, 1% casamino acid, 0.2% yeast extract, 0.2% $KH_2PO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$.

The $a_w$ of the media were measured at 25°C using an electric hygrometer, Hygroskop DT (Rotronic, Switzerland).

*Incubation.* All the strains were preincubated on YM agar medium for 2 or 3 days. The yeast cells were suspended in sterilized 0.87% NaCl solution and inoculated into broths with different $a_w$. The initial cell concentration was $10^2$/ml. The inoculated broths were incubated at 25°C for up to 120 days. During incubation, the growth of yeasts was examined by observing the turbidity of broths and counting yeast cells by the plating method.

Five strains of Z. *rouxii* preincubated on YM agar were also incubated in Medium A, Medium B, and Medium C to study the effects of nutrients in media.

Several strains were preincubated on agar media containing 50% (w/w) glucose or 40% (w/w) fructose, and incubated in the same ways to examine the effects of preincubation in the presence of high concentrations of $a_w$-controlling solutes.

## RESULTS

The relationships between the concentrations of four kinds of solutes (expressed as g per 100 g solutions) and the $a_w$ of media containing these solutes are shown in Fig. II-1. Sodium chloride

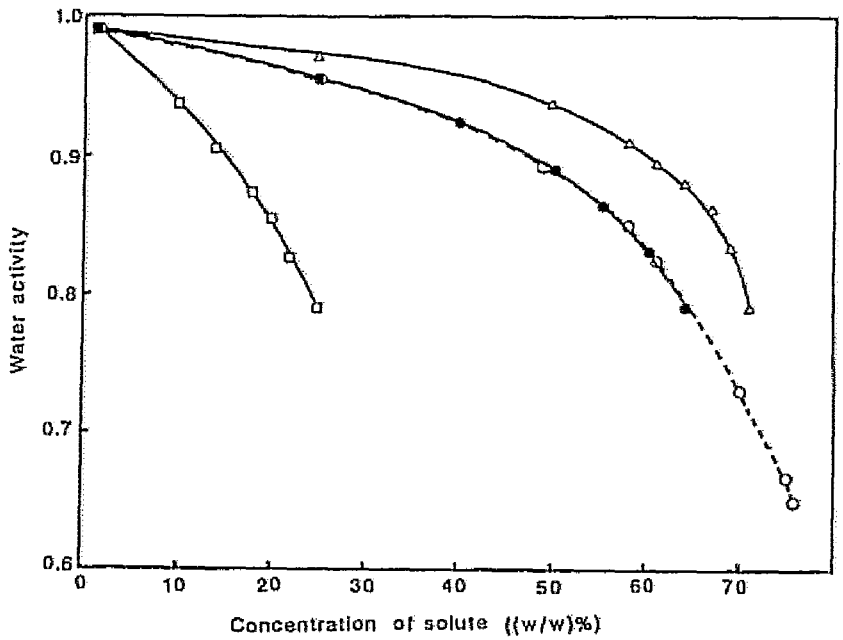


Fig. II-1 Relationships between the $a_w$ of media and the concentrations of $a_w$-controlling solutes.

Media contained 1% glucose, 0.5% polypeptone, 0.3% yeast ext., 0.3% malt ext., and different concentrations of $a_w$-controlling solutes.

●: glucose-media, ○: fructose-media, △: sucrose-media, □: NaCl-media.

was the most effective solute for decreasing $a_w$ because of its small molecular weight and dissociation. On the other hand, sucrose was the least effective.

Glucose and sucrose have lower solubility in water than fructose and the lowest $a_w$ of broth to which they could adjust was 0.79 (64% (w/w) glucose or 71% (w/w) sucrose), while fructose could adjust the $a_w$ of broth to a level as low as 0.65 (76% (w/w) fructose).

Table II-1 shows the minimum $a_w$ for growth of 35 strains of yeasts isolated from high-sugar foods. They differed significantly from species to species. There were also differences among the minimum $a_w$ for growth of five strains of *Z. rouxii*.

The effects of $a_w$-controlling solutes on the minimum $a_w$ for the growth of each strain were observed. In most species, incubation in broths containing sodium chloride as the $a_w$-controlling solute (NaCl-media) gave higher minimum $a_w$ for growth than those in broths containing glucose, fructose, or sucrose (glucose-, fructose-, or sucrose-media). Only a few species, such as *D. hansenii* and *H. anomala*, showed similar minimum $a_w$ for growth in NaCl-media and three kinds of sugar-media.

The effects of three kinds of sugars on minimum $a_w$ for growth differed from species to species. About half of the species showed the lowest minimum $a_w$ for growth in sucrose-media and about one third showed the lowest values in glucose-media and sucrose-media.

The strain which showed the lowest $a_w$ for growth (0.67) was *Z. rouxii* 38-40A. This was shown in fructose-media. *Z. bisporus*, *C. apicola*, *C. bombi*, *C. glucosophila*, *C. lactiscondensi*, *C. mannitofaciens*, *C. nodaensis*, and *C. versatilis* also had minimum $a_w$ for growth lower than 0.79.

Table II-2 shows the minimum $a_w$ for the growth of five strains of *Z. rouxii* in four kinds of media. The minimum $a_w$ for the growth of the two strains 88-40A and 151-40A, isolated from high-sugar foods, significantly decreased in Media A, B, and C. Medium B was particularly effective. But the effects on the $a_w$ of strain 38-40A were small. The minimum $a_w$ for the growth of the two strains 86-A and 87-A, isolated from soy sauce and miso, respectively, decreased only in Media A and C.

Tables II-3 and II-4 show the effects of preincubation in the presence of high concentrations of sugars on the minimum $a_w$ for the growth of several strains. Preincubation on agar media containing 50% (w/w) glucose or 40% (w/w) fructose decreased the minimum $a_w$ for growth in glucose-media or fructose-media of all strains examined.

Table II-1 Some characteristics and minimum $a_w$ for growth of

| Strain | | Source | Assimilation | |
|---|---|---|---|---|
| | | | Glucose | Fructose |
| *Debaryomyces hansenii* | 67-40A | blueberry sauce | + | + |
| *Hansenula anomala* | 150-40A | sponge cake | + | + |
| *Pichia ohmeri* | 19-40A | flower | + | + |
| *Pichia membranaefaciens* | 114-CA | soil | + | + |
| *Saccharomyces cerevisiae* | 89-25A | candied apple | + | + |
| *Schizosaccharomyces pombe* | 57-50B | molasses | + | + |
| *Torulaspora delbrueckii* | 154-CA | sweet bean paste | + | + |
| *Torulaspora globosa* | 26-40A | papaya | + | + |
| *Zygosaccharomyces bisporus* | 153-40A | molasses | + | + |
| *Zygosaccharomyces rouxii* | 38-40A | honey | + | + |
| *Zygosaccharomyces rouxii* | 86-A | soy sauce | + | + |
| *Zygosaccharomyces rouxii* | 87-A | miso | + | + |
| *Zygosaccharomyces rouxii* | 88-40A | candied cherry | + | + |
| *Zygosaccharomyces rouxii* | 151-40A | molasses | + | + |
| *Candida apicola* | 22-40A | apple | + | + |
| *Candida bombi* | 19-25A | flower | + | + |
| *Candida bombicola* | 34-40B | flower | + | + |
| *Candida dattila* | 128-25A | raisin | + | + |
| *Candida dulciaminis* | 155-CA | sponge cake | + | + |
| *Candida famata* | 147-40A | sap | + | + |
| *Candida glucosophila* | 29-25A | brown sugar | + | + |
| *Candida guilliermondii* | 22-25A | apple | + | + |
| *Candida intermedia* | 147-CA | sap | + | + |
| *Candida lactiscondensi* | 91-40A | angelica | + | + |
| *Candida lusitaniae* | 91-CA | angelica | + | + |
| *Candida mannitofaciens* | 152-40A | molasses | + | + |
| *Candida nodaensis* | 24-60A | kiwi fruit | + | + |
| *Candida oregonensis* | 107-40A | flower | + | + |
| *Candida tropicalis* | 118-40A | lemon cake | + | + |
| *Candida versatilis* | 24-60B | kiwi fruit | + | + |
| *Kloeckera apis* | 26-50A | papaya | + | + |
| *Rhodotorula glutinis* | 13-40A | flower | + | + |
| *Rhodotorula rubra* | 70-25A | jam | + | + |
| *Rhodosporidium* sp. | 65-25A | dried date | + | + |
| *Sympodiomycopsis paphiopedili* | 101-40A | nectar | + | + |

+: positive, −: negative, w: weak.

## DISCUSSION

The results in Fig. II-1 agree with the previous reports. Kushner (8) showed the relationship between the concentrations of different solutes (expressed as percent (w/v)) and $a_w$ of these solutions. Since the concentrations of solutes were expressed as percent (w/w) in this study, the dependences of $a_w$ on solute concentrations were more drastic.

yeasts isolated from high-sugar foods and related materials.

| Assimilation | Minimum $a_w$ for growth in | | | |
|---|---|---|---|---|
| Sucrose | Glucose-media | Fructose-media | Sucrose-media | NaCl-media |
| + | 0.84 | 0.86 | 0.81 | 0.84 |
| + | 0.86 | 0.91 | 0.84 | 0.84 |
| + | 0.84 | 0.86 | 0.83 | 0.90 |
| − | 0.90 | 0.92 | 0.90 | 0.94 |
| + | 0.89 | 0.91 | 0.89 | 0.92 |
| + | 0.84 | 0.86 | 0.87 | 0.95 |
| + | 0.86 | 0.89 | 0.87 | 0.90 |
| + | 0.86 | 0.89 | 0.85 | 0.90 |
| − | 0.85 | 0.85 | ≦0.79 | 0.95 |
| − | ≦0.79 | 0.67 | ≦0.79 | 0.95 |
| − | ≦0.79 | 0.73 | ≦0.79 | 0.92 |
| − | ≦0.79 | 0.83 | ≦0.79 | 0.95 |
| − | 0.81 | 0.82 | ≦0.79 | 0.88 |
| w | ≦0.79 | 0.82 | ≦0.79 | 0.86 |
| + | ≦0.79 | 0.79 | ≦0.79 | 0.90 |
| + | ≦0.79 | 0.83 | ≦0.79 | 0.90 |
| + | 0.84 | 0.85 | 0.85 | 0.92 |
| + | 0.88 | 0.91 | 0.87 | 0.90 |
| + | 0.84 | 0.91 | 0.81 | 0.86 |
| + | 0.91 | 0.92 | 0.90 | 0.94 |
| − | 0.81 | 0.85 | ≦0.79 | 0.83 |
| + | 0.89 | 0.89 | 0.85 | 0.88 |
| + | 0.90 | 0.92 | 0.89 | 0.92 |
| + | ≦0.79 | 0.78 | ≦0.79 | 0.92 |
| + | 0.90 | 0.92 | 0.87 | 0.90 |
| + | ≦0.79 | 0.73 | ≦0.79 | 0.86 |
| − | 0.81 | 0.80 | ≦0.79 | 0.82 |
| + | 0.86 | 0.89 | 0.85 | 0.90 |
| + | 0.88 | 0.88 | 0.83 | 0.90 |
| + | ≦0.79 | 0.80 | ≦0.79 | 0.84 |
| − | 0.90 | 0.93 | 0.90 | 0.95 |
| − | 0.89 | 0.93 | 0.89 | 0.92 |
| + | 0.90 | 0.92 | 0.90 | 0.90 |
| + | 0.89 | 0.91 | 0.89 | 0.94 |
| + | 0.89 | 0.91 | 0.85 | 0.90 |

Several species belonging to the genus *Candida* such as *C. apicola* and *C. bombicola* showed low minimum $a_w$ for growth in sugar-media, although most of previous papers described low minimum $a_w$ for the growth of only the genus *Zygosaccharomyces*.

Three strains of *Z. rouxii* isolated from high-sugar foods were only sugar-tolerant, while two strains of *Z. rouxii* isolated from high-salt foods such as soy sauce and miso were both sugar-tolerant and salt-tolerant. These results agree with the report

Table II-2 Comparison of minimum $a_w$ for growth of *Zygosaccharomyces rouxii* in four kinds of NaCl-media.

| Strain | Minimum $a_w$ for growth in | | | |
|---|---|---|---|---|
| | YM+NaCl | Medium A | Medium B | Medium C |
| *Zygosaccharomyces rouxii* 38-40A | 0.95 | 0.95 | 0.93 | 0.94 |
| *Zygosaccharomyces rouxii* 86-A | 0.88 | 0.86 | 0.88 | 0.85 |
| *Zygosaccharomyces rouxii* 87-A | 0.86 | 0.82 | 0.86 | 0.85 |
| *Zygosaccharomyces rouxii* 88-40A | 0.92 | 0.86 | 0.84 | 0.89 |
| *Zygosaccharomyces rouxii* 151-40A | 0.95 | 0.92 | 0.86 | 0.89 |

Medium A: 90 ml of YM broth, 10 ml of miso ext., and NaCl.
Medium B: 90 ml of koji ext., 10 ml of raw soy sauce, 0.5% glucose, and NaCl.
Medium C: 5% glucose, 1% casamino acid, 0.2% yeast ext., 0.2% $KH_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, and NaCl.

Table II-3 Effects of pre-incubation in the presence of high concentrations of glucose on minimum $a_w$ for growth of yeasts.

| Strain | | Pre-incubation on | |
|---|---|---|---|
| | | YM | 50% (w/w) glucose-medium |
| *Debaryomyces hansenii* | 67-40A | 0.86 | 0.80 |
| *Torulaspora delbrueckii* | 154-CA | 0.86 | 0.80 |
| *Zygosaccharomyces rouxii* | 38-40A | 0.85 | 0.80 |
| *Candida bombicola* | 34-40B | 0.86 | 0.80 |
| *Candida nodaensis* | 24-60A | 0.83 | 0.75 |

Table II-4 Effects of pre-incubation in the presence of high concentrations of fructose on minimum $a_w$ for the growth of yeasts.

| Strain | | Pre-incubation on | |
|---|---|---|---|
| | | YM | 40% (w/w) fructose-medium |
| *Hansenula anomala* | 150-40A | 0.89 | 0.85 |
| *Torulaspora delbrueckii* | 154-CA | 0.90 | 0.85 |
| *Candida dattila* | 128-25A | 0.91 | 0.86 |
| *Candida lusitaniae* | 91-CA | 0.91 | 0.87 |
| *Candida oregonensis* | 107-40A | 0.91 | 0.87 |
| *Rhodotorula glutinis* | 13-40A | 0.91 | 0.87 |

by Onishi (9) that the salt-tolerance and sugar-tolerance of *Z. rouxii* differ considerably, strain by strain, according to their origin. The fact that most of the yeast strains isolated from high-sugar foods showed lower minimum $a_w$ for growth in sugar-

media compared with NaCl-media indicated that the mechanism of sugar-tolerance differs from that of salt-tolerance. Onishi (10) found that the differences between sugar-tolerance and salt-tolerance were also observed in pH ranges for growth. Inhibition of microbial growth by sodium chloride could be caused by inhibition of enzyme activities, decrease in solubilities of oxygen, increased sensitivities of cells to carbon dioxide, the toxic action of chrome ions, and so on.

One factor in the differences of minimum $a_w$ for growth in the presence of different sugars could be a difference between the mechanism of glucose-tolerance and that of sucrose-tolerance. Moran and Witter (11) found that production of D-arabitol increased when *Z. rouxii* (*Saccharomyces rouxii*) cells were grown in the presence of increasing glucose concentrations, while growth in high concentrations of sucrose had no effect on the production of D-arabitol. Another factor could be a difference between the protective action of sugars on yeast cells. Sucrose was likely to have the largest action to protect yeast cells among three kinds of sugars, because yeast cells were alive even below minimum $a_w$ for growth in sucrose-media, although they died out below the minimum $a_w$ for growth in glucose-media and fructose-media. This action of sucrose decreased the minimum $a_w$ for growth in the strains which did not assimilate sucrose as well as in the strains which did assimilate it. This shows that the ability or inability of yeasts to utilize carbon compounds does not always contribute to such decreases of minimum $a_w$ for growth.

The minimum $a_w$ for growth are affected by other environmental factors such as temperature, pH, concentration of oxygen and carbon dioxide, and nutrients; they are the lowest when these factors are most suitable for microbial growth. This study was carried out under almost the most suitable conditions. Sato et al. (12) and Onishi (10) reported that *Z. rouxii* required vitamins such as inositol, panthotenic acid, and biotin in the presence of sodium chloride. Kato et al. (13) found that soy sauce markedly accelerated the growth of osmophilic yeasts and identified the effective component as myo-inositol. We examined the effects of miso extract and koji extract containing raw soy sauce, which were rich in inositol, and casamino acid as a nitrogen source on the minimum $a_w$ for the growth of *Z. rouxii*. Results in Table II-2 support the previous papers describing acceleration of growth by inositol and demonstrate that casamino acid also decreased minimum $a_w$ for growth.

The fact that preincubation in the presence of high concentrations of sugars decreased the minimum $a_w$ for growth

showed that the osmotolerance of the yeast cells were affected by adaptation to environment, although this is generally considered to be genetic. This agreed with a previous paper describing the decrease in survival of cells by transporting cells to media containing considerably different concentrations of sodium chloride (14).

Some strains tested in this study had low minimum $a_w$ for growth below 0.80. This suggests that combinations of low $a_w$ with other environmental factors such as lower temperatures and lower concentrations of oxygen are important for preventing spoilage by yeasts in foods with intermediate moisture.

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# CHAPTER III

## CHARACTERIZATION OF SUGAR-TOLERANT YEASTS

As described in introduction, microorganisms having the ability to thrive in environments with low water activity ($a_w$) include halophilic and halotolerant bacteria, halophilic algae, sugar-and salt-tolerant yeasts, xerophilic fungi, and so on. The enzymes of halophilic bacteria are intrinsically distinctive and require sodium chloride or potassium chloride for activation and stabilization (1). These bacteria maintain intracellular concentrations of sodium and potassium ions at levels at least as high as those of external concentrations (2, 3). The enzymes of halophilic algae are not distinctive and are sharply inhibited by sodium chloride (4). These algae accumulate glycerol in response to increasing environmental salinity (5), and glycerol is thought to act as a compatible solute that osmoregulates and protects enzymes (6). Osmoregulation of nonhalophilic bacteria is reported to be achieved by the accumulation of both potassium ions and such amino acids as prolin and glutamate (7, 8).

Salt-tolerant yeasts have been reported to accumulate such polyols as glycerol and D-arabitol in response to increasing sodium chloride concentration (9-14), and these polyols are believed to act as compatible solutes (10, 11). The importance of the role of glycerol was emphasized in the process of salt-tolerance of *Z. rouxii* and *D. hansenii* (11, 13, 14). Lipid compositions (15-19), cell wall composition (20-22), and $Na^+/K^+$-activated ATPase and cell membrane bound $H^+$-ATPase (23-25) of salt-tolerant yeasts were also studied.

Sugar-tolerance of yeasts are less extensively studied than salt-tolerance of yeasts. This chapter deals with the characteristics of sugar-tolerant yeasts described in CHAPTER I and II.

### III-1. Intracellular solutes contributing to osmoregulation

Brown (26, 27) reported that sugar-tolerance of *Z. rouxii* was achieved by accumulation of D-arabitol as a compatible solute. On the other hand, Moran and Witter (28) suggested that the active pentose phosphate pathway was involved in glucose-tolerance of *Z. rouxii* and that D-arabitol might serve as a fermentation end product. Thus, the relationships between the accumulation of polyols in sugar-tolerant yeasts and the mechanisms of their tolerance had not been clarified in the previous studies. The author investigated intracellular solutes contributing to

osmoregulation in some sugar-tolerant yeasts and their relations to the sugar-tolerance of yeasts.

## MATERIALS AND METHODS

*Strains.* Seven yeast strains, *D. hansenii* 67-40A, *H. anomala* 150-40A, *S. cerevisiae* 89-25A, *T. delbrueckii* 154-CA, *Z. rouxii* 88-40A, *C. tropicalis* 118-40A, and *Aureobasidium* sp. SN-G42 (29) were examined.

*Growth media and cultivation.* Forty-five percent (w/w) glucose, 56% (w/w) sucrose, and 8, 10, or 12% (w/w) NaCl were added to YM broth as a basal medium for preparing 45% (w/w) glucose medium, 56% (w/w) sucrose medium, and 8, 10, or 12% (w/w) NaCl medium, respectively. Three kinds of media containing 45% (w/w) glucose, 56% (w/w) sucrose, and 12% (w/w) NaCl had $a_w$ of about 0.91. NaCl concentrations of 8 and 10 % (w/w) were used for the strains that could not grow in 12% (w/w) NaCl medium. After preincubation in 5 ml of YM broth at 30°C, the cultures were inoculated into 200 ml of four kinds of broth. During incubation on a rotary shaker at 30°C, about 40 ml of the cultures were taken at constant intervals. The cultured cells were collected by centrifugation at 3,500 *xg* for 10 min, and rapidly washed twice with 0.87% NaCl solution.

*Transfer experiments.* Two strains, *D. hansenii* 67-40A and *C. tropicalis* 118-40A, were incubated in 200 ml of YM broth at 30°C and the late logarithmic phase cells were collected by centrifugation. All of the cells were inoculated into 200 ml of four kinds of broth described above. They were incubated at 30°C for 15 to 20 hr, and during incubation, sampling of the cells was carried out as described above.

*Extraction of intracellular solutes.* Sample cells were freeze-dried and intracellular solutes were extracted in 80% (v/v) aqueous ethanol for 1 hr, using inositol as an internal standard. The extracts were filtered to remove cells, followed by repeating extraction two more times under the same conditions. After evaporating the extraction solvent, samples were filled up to 10 ml with distilled water and divided into two equal parts. The sample solutions were stored at -20°C until analysis.

*Determination of free amino acids.* Five ml of the sample solutions were dried up and dissolved into 1 ml of 0.2 N lithium citrate buffer (pH 2.2). They were filtrated through a membrane filter with a pore size of 0.45 μm (Nihon Millipore Kogyo K.K.) and 50 μl of the filtrates were injected into an amino acid analyzer (Hitachi 835).

*Determination of sugars and polyols.* 0.5 ml of the sample

solutions were completely dried up, and then derivatized by adding 0.2 ml pyridine containing 0.5% *n*-cetane as an internal standard, 0.2 ml 1,1,1,3,3,3-hexamethyl-disilazane, and 0.1 ml trifluoroacetic acid and mixing vigorously for 20 sec. After standing for 10 min, the derivatized samples were separated and determined using a gas chromatograph (Shimadzu GC-14A) equipped with a packed column of Silicone SE-52. For determining the amounts of extracellular polyols and sugars, 0.1 ml of culture supernatants were used.

## RESULTS AND DISCUSSION

There was no significant effect of solute concentration of media on the amounts and compositions of intracellular free amino acids in the tested strains (data not shown). The amounts of intracellular polyols and sugars in seven strains grown in four kinds of media are shown in Figs III-1 - III-7. All the tested strains accumulated glycerol at the initial stage of cultivation. In four strains, *D. hansenii* 67-40A, *H. anomala* 150-40A, *Z. rouxii* 88-40A, and *C. tropicalis* 118-40A, D-arabitol increased

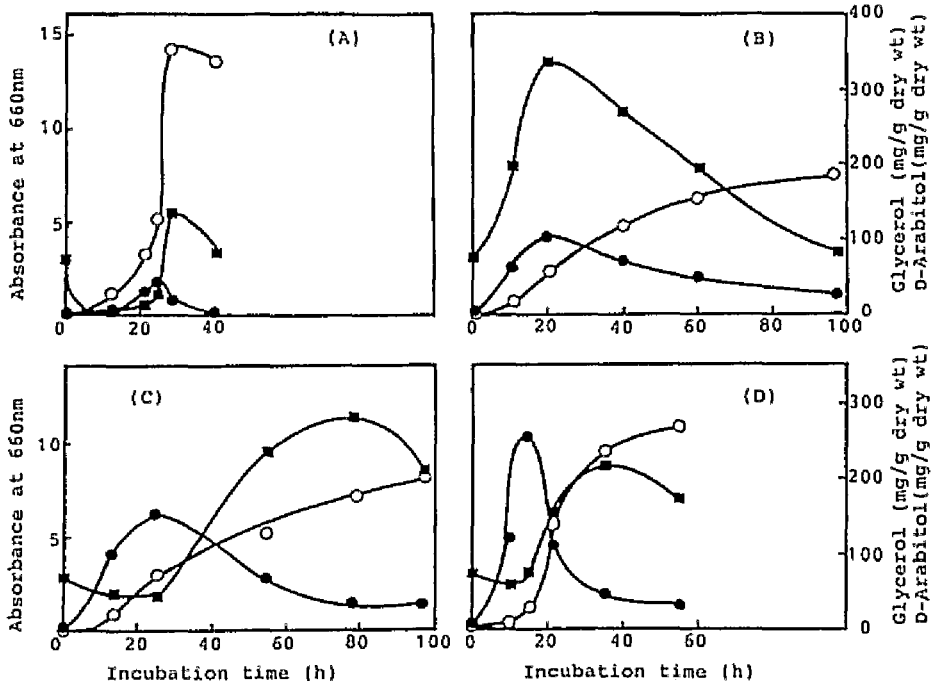


Fig. III-1 Intracellular concentrations of polyols and sugars in *Debaryomyces hansenii* 118-40A grown in low- and high-solute media.

(A) YM medium, (B) 45% (w/w) glucose medium, (C) 56% (w/w) sucrose medium, (D) 12% (w/w) NaCl medium. Symbols: O, absorbance at 660nm; ●, glycerol; ■, D-arabitol.

as glycerol decreased, with a few exceptions (Figs III-1 - III-4). *Aureobasidium* sp. SN-G42 accumulated erythritol instead of D-arabitol (Fig. III-5). *S. cerevisiae* 89-25A accumulated neither D-arabitol nor erythritol, and trehalose increased with the decrease in glycerol (Fig. III-6). *T. delbrueckii* 154-CA belonged to an intermediate type, which accumulated both D-arabitol and trehalose (Fig. III-7).

In the two strains of *Z. rouxii* 88-40A and *C. tropicalis* 118-40A, there was accumulation of larger amounts of glycerol in NaCl-medium compared with glucose- and sucrose-media. In *T. delbrueckii* 154-CA, accumulation of high concentrations of polyols, in particular D-arabitol, was observed in sucrose-medium, but not in glucose- and NaCl-media. These results suggest that there are differences between glucose-tolerance and sucrose-tolerance of yeasts as well as between sugar-tolerance and salt-tolerance. Such results agreed with the previous studies on *Z. rouxii* (28, 30), although Moran and Witter (28) reported that accumulation of D-arabitol was observed only in the presence of high concentrations of glucose. It is suggested that sugar-tolerance can not be understood solely by osmoregulation by

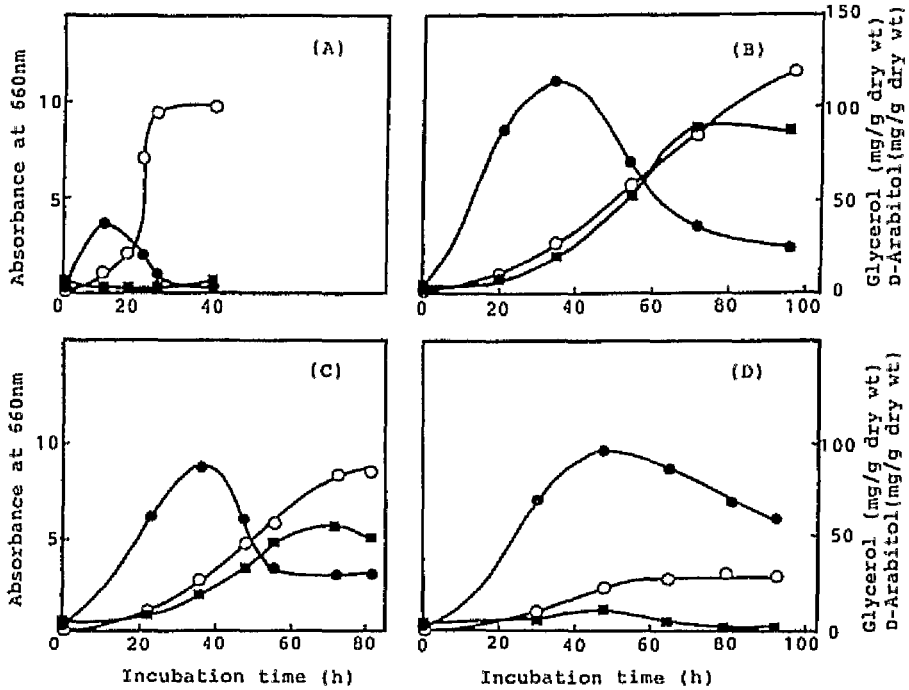


Fig. III-2 Intracellular concentrations of polyols and sugars in *Hansenula anomala* 150-40A grown in low- and high-solute media.

(A) YM medium, (B) 45% (w/w) glucose medium, (C) 56% (w/w) sucrose medium, (D) 12% (w/w) NaCl medium. Symbols: O, absorbance at 660nm; ●, glycerol; ■, D-arabitol.

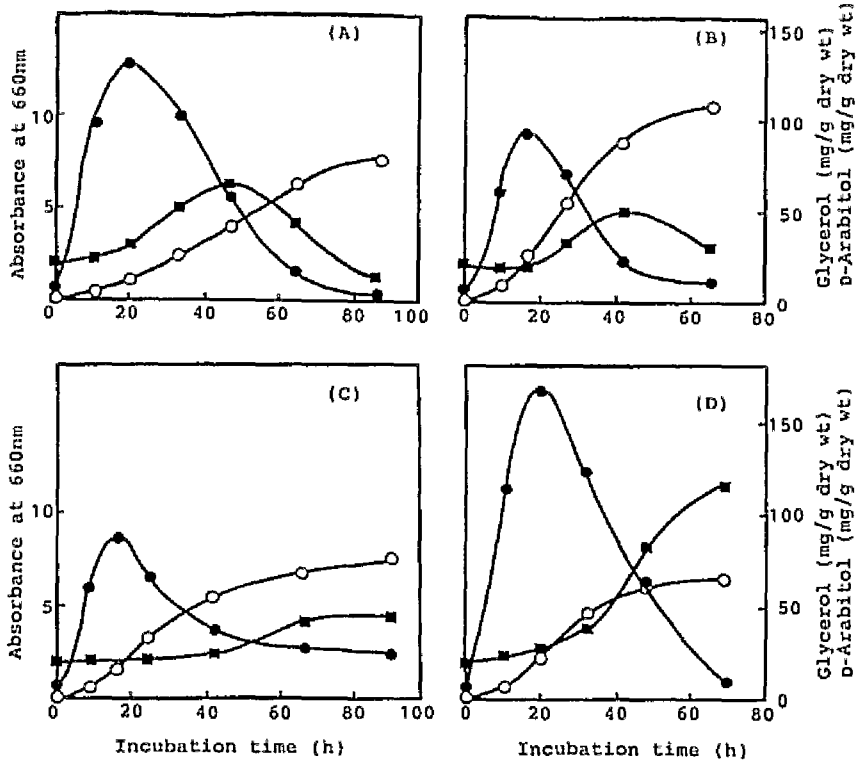


Fig. III-3 Intracellular concentrations of polyols and sugars in *Zygosaccharomyces rouxii* 88-40A grown in low- and high-solute media.

(A) YM medium, (B) 45% (w/w) glucose medium, (C) 56% (w/w) sucrose medium, (D) 10% (w/w) NaCl medium. Symbols: O, absorbance at 660nm; ●, glycerol; ■, D-arabitol.

intracellularly accumulating polyols. It is necessary to further investigate whether other solutes may accumulate and whether other mechanisms may be involved in sugar-tolerance.

In most strains, higher concentrations of intracellular polyols accumulated in glucose-, sucrose- and NaCl-media compared with that in YM medium, although *Z. rouxii* 88-40A showed higher concentrations of polyols in YM medium compared to glucose- and sucrose-media. Significant differences in the maximum concentrations of polyols between YM medium and the other media were observed in *D. hansenii* 67-40A and *H. anomala* 150-40A. However, the accumulation of polyols in the high-solute media was temporary and decreased during the late logarithmic period of cultivation.

This decrease was shown to be due to the release of polyols into cultivation broth, based on the results on the amounts of extracellular polyols determined for *Aureobasidium* sp. SN-G42 (Fig. III-5). In the high-solute media, extracellular glycerol increased with the growth of this strain, followed by the

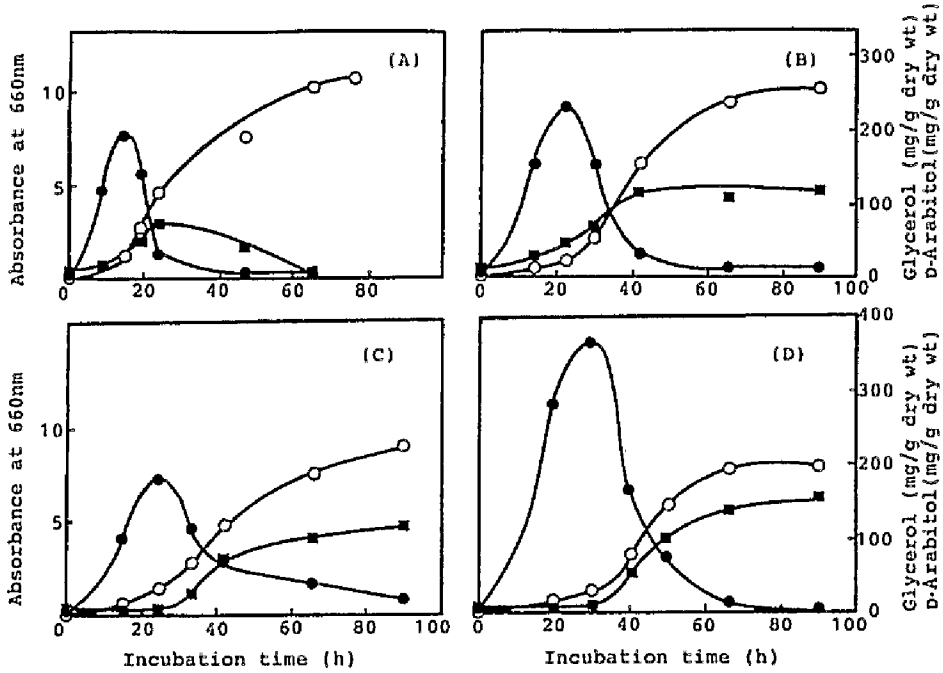


Fig. III-4 Intracellular concentrations of polyols and sugars in *Candida tropicalis* 118-40A grown in low- and high-solute media.

(A) YM medium, (B) 45% (w/w) glucose medium, (C) 56% (w/w) sucrose medium, (D) 12% (w/w) NaCl medium. Symbols: O, absorbance at 660nm; ●, glycerol; ■, D-arabitol.

increase in extracellular erythritol. Only small amounts of extracellular glycerol were detected in YM medium.

The maximum concentrations of intracellular glycerol in the tested strains grown in the high-solute media ranged from 70 to 370 mg/g of dry cell weight. Those of D-arabitol, erythritol, and trehalose were 50-400, 60-130, and 15-55 mg/g of dry cell weight, respectively. The maximum total concentration of these intracellular solutes, observed in *C. tropicalis* 118-40A grown in NaCl-medium (Fig. III-4 (D)) and *T. delbrueckii* 154-CA grown in sucrose-medium (Fig. III-7 (C)), was about 1 M. This value was calculated on the basis of the fact that the water content of the tested yeasts was approximately 80%. This is about 30% of the molarity of glucose-medium and about 40% that of sucrose- and NaCl-media, and therefore it is not always sufficient to balance the osmotic pressure of media. On the other hand, it was reported that the intracellular polyol concentration was sufficient to balance about 75% of the osmotic pressure of media with lower NaCl concentrations of 0.68 and 1.35 M (14).

These results suggest that such intracellular polyols as glycerol and D-arabitol could contribute to the osmoregulation of sugar-tolerant yeasts, although they may not always be produced

for the purpose of osmoregulation. In the study on osmotic regulation in algae and higher plants by Schobert (31), a hypothesis was proposed that accumulating polyols can replace water molecules by means of the polyols' waterlike OH-groups and thus participate in the hydrophobically enforced water structure. This mechanism was thought to result in the maintenance of complete hydration of biopolymers, even with a reduced number of available water molecules. Thus, the possibility of such a mechanism could be supposed in the case of intracellular polyols of sugar-tolerant yeasts.

Changes in the amount of intracellular polyols and sugars during the induction period after transfer from YM medium into four kinds of media were examined, using *D. hansenii* 67-40A and *C. tropicalis* 118-40A (Figs III-8 and III-9). Intracellular glycerol of both strains transferred to YM medium increased within 2 hr after transfer, but then gradually decreased to initial concentrations. In contrast, transfer to three high-solute media caused the increase in intracellular glycerol by 5.5-18 times within 20 hr. In particular, significant increases

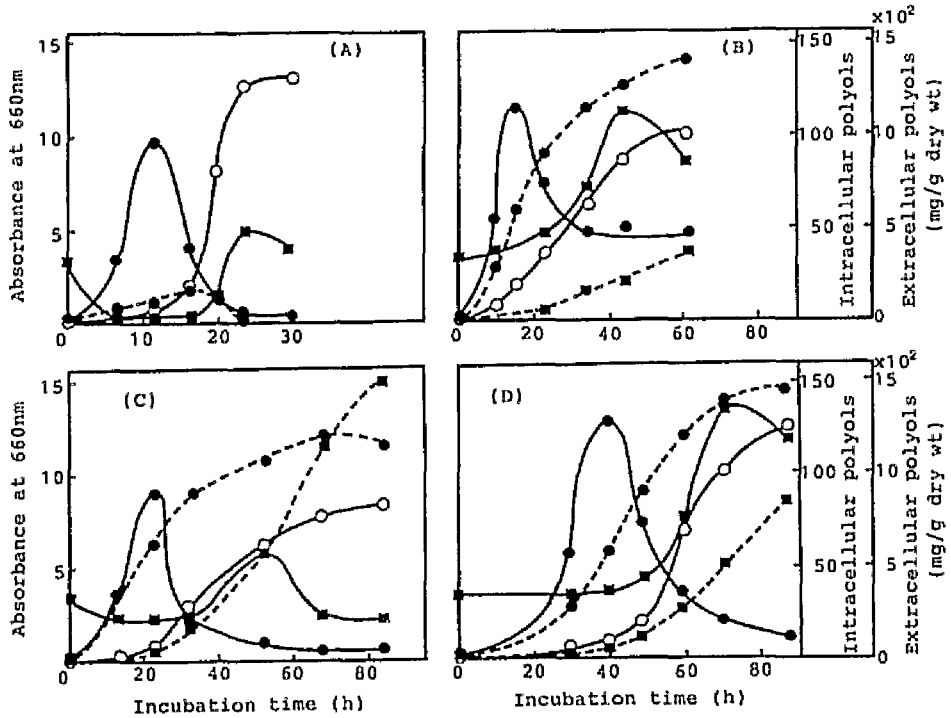


Fig. III-5 Intracellular and extracellular concentrations of polyols and sugars in *Aureobasidium* sp. SN-G42 grown in low- and high-solute media.

(A) YM medium, (B) 45% (w/w) glucose medium, (C) 56% (w/w) sucrose medium, (D) 10% (w/w) NaCl medium. Symbols: O, absorbance at 660 nm; —●—, intracellular glycerol; —■—, intracellular erythritol; --●--, extracellular glycerol; --■--, extracellular erythritol.

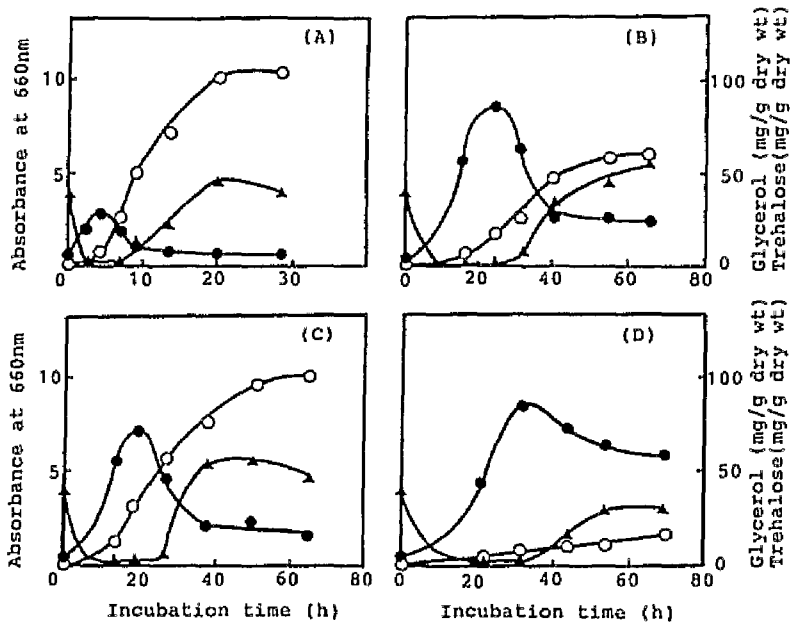


Fig. III-6 Intracellular concentrations of polyols and sugars in *Saccharomyces cerevisiae* 89-25A grown in low- and high-solute media.

(A) YM medium, (B) 45% (w/w) glucose medium, (C) 56% (w/w) sucrose medium, (D) 8% (w/w) NaCl medium. symbols: O, absorbance at 660 nm; ●, glycerol; ▲, trehalose.

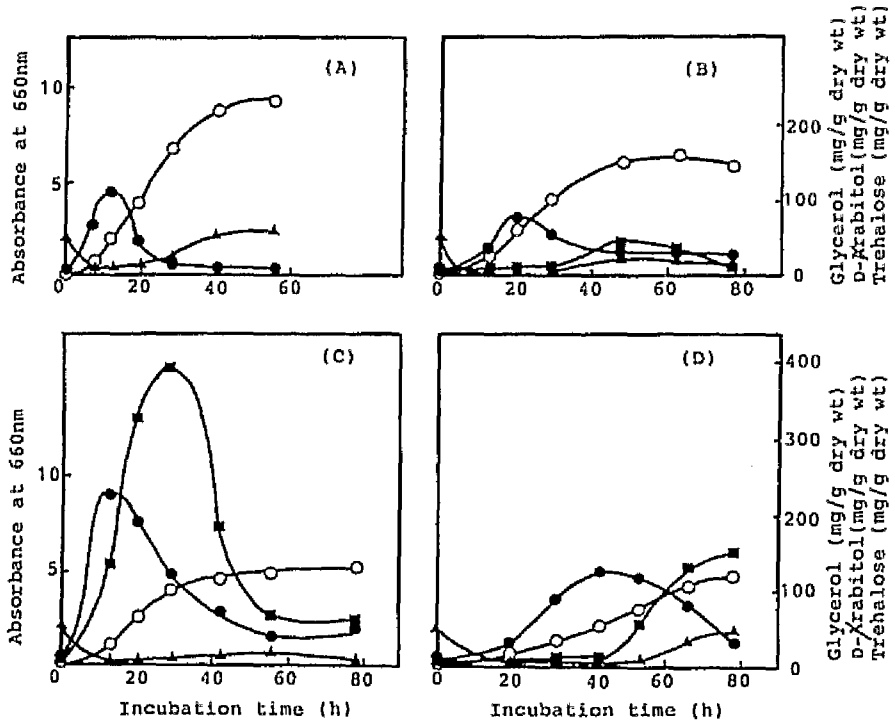


Fig. III-7 Intracellular concentrations of polyols and sugars in *Torulaspora delbrueckii* 154-CA grown in low- and high-solute media.

(A) YM medium, (B) 45% (w/w) glucose medium, (C) 56% (w/w) sucrose medium, (D) 12% (w/w) NaCl medium. Symbols: O, absorbance at 660nm; ●, glycerol; ■, D-arabitol; ▲, trehalose.

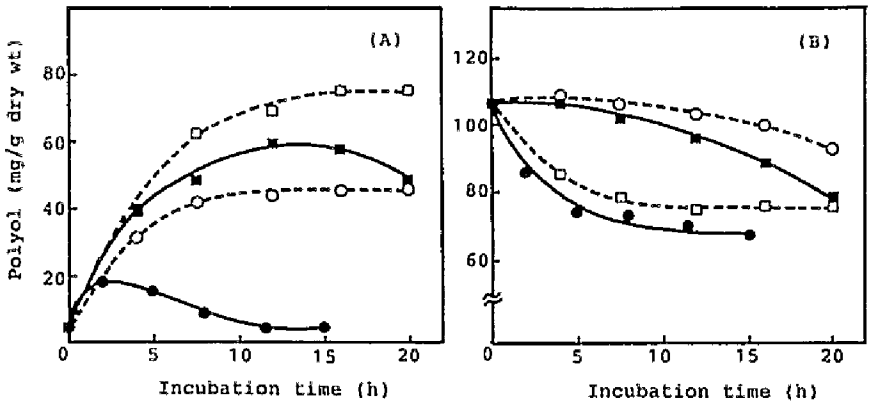


Fig. III-8 Changes in intracellular concentration of polyols in *Debaryomyces hansenii* 67-40A after transfer to low- and high-solute media.

(A) Glycerol, (B) D-arabitol. Symbols: —●—, YM medium; —■—, glucose medium; --○--, sucrose medium; --□--, NaCl medium.

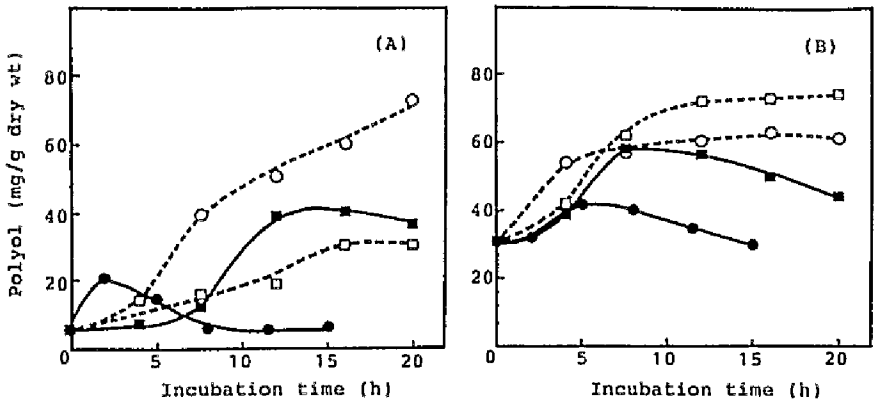


Fig. III-9 Changes in intracellular concentration of polyols in *Candida tropicalis* 118-40A after transfer to low- and high-solute media.

(A) Glycerol, (B) D-arabitol. Symbols: —●—, YM medium; —■—, glucose medium; --○--, sucrose medium; --□--, NaCl medium.

were observed in *D. hansenii* cells in NaCl-medium and *C. tropicalis* cells in sucrose-medium. Changes in D-arabitol concentration were much smaller compared with those in glycerol concentration. In *C. tropicalis*, changes in D-arabitol concentration showed patterns similar to those in glycerol concentration, whereas the concentration decreased in *D. hansenii*. These results suggest that glycerol may be an important intracellular solute for osmoregulation, as reported in the previous papers on salt-tolerance of yeasts (11, 13, 14).

## III-2. Purification and properties of erythrose reductase from an *Aureobasidium* sp. mutant

*Aureobasidium* sp. SN-G42 (29), one of the strains used for commercial production of erythritol, is extremely sugar-tolerant; it can grow in media containing 64% (w/w) glucose or 71% (w/w) sucrose. This strain produces a large amount of erythritol intracellularly at first and then extracellularly as described in III-1. Erythritol was found to be synthesized from erythrose 4-phosphate, an intermediate of the pentose-phosphate cycle, by dephosphorylation followed by reduction of the resultant erythrose. Erythrose reductase, catalyzing this last step, was found to be a key enzyme. This section describes purification and properties of erythrose reductase from *Aureobasidium* sp. SN-G42.

### MATERIALS AND METHODS

*Microorganism and cultivation.* *Aureobasidium* sp. SN-G42 (29), a mutant from *Aureobasidium* sp. SN-124A, was cultured in 30 liters of liquid medium containing 40% (w/v) glucose and 2% yeast extract in 2 sets of 30-liter jar-fermentor at 35°C for 40 hr. The cells were collected by centrifugation and freeze-dried.

*Assay of enzymatic activity.* Reductive activity of erythrose reductase was assayed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADPH. Under the standard reaction conditions, 5 µl of the enzyme solutions was incubated in 250 µl of 50 mM phosphate buffer (pH 6.5) containing 12 mM D-erythrose and 4 mM NADPH at 37°C for 10 min. Immediately after the reaction was stopped by adding 2 ml of 0.2 M $Na_2HPO_4$-NaOH buffer (pH 11) to the reaction mixture, the absorbance at 340 nm was measured by a Shimadzu UV-160A spectrophotometer. One unit (U) of the activity was defined as the amount of enzyme that produced 1 µmol of $NADP^+$ per minute under the above conditions. Oxidative activity of this enzyme was measured by the increase in absorbance at 340 nm after incubating 10 µl of the enzyme solution in 250 µl of 50 mM $Na_2HPO_4$ - NaOH buffer (pH 9.5) containing 80 mM polyol and 5 mM $NADP^+$ at 37°C for 10 hr.

*Measurement of protein.* Protein was measured by Bradford method (32) with bovine serum albumin as the standard. Protein in the column elutes was routinely monitored by the absorbance at 280 nm.

*Polyacrylamide gel electrophoresis (PAGE).* PAGE in the absence (native) or presence of sodium dodecyl sulfate (SDS) and isoelectric focusing (IEF) PAGE were done mainly on the Phastsystem of Pharmacia LKB Biotechnology according to their

manual. PhastGel (Gradient 8-25) was used for native and SDS-PAGE, and PhastGel IEF pH 4-6.5 for IEF-PAGE. Protein on the electrophoresed gels was stained using a PhastGel Silver Kit from Pharmacia. For activity staining, the electrophoresed gel plate was incubated at 37°C for 15 min in 0.2 M boric acid - NaOH buffer (pH 9.5) containing 1% erythritol, 0.05% $NADP^+$, 0.03% tetranitro blue tetrazolium (TNBT), and 0.004% phenazine methosulfate (PMS). Then the gel was washed with distilled water and stored in 0.8% acetic acid solution.

*Gel filtration.* Gel filtration by HPLC was done on an Asahi-Pack GS-510 column (7.6 x 500 mm), which was equilibrated with 0.2 M phosphate buffer (pH 7.0). The elution was done at a rate of 1.0 ml/min.

## RESULTS

*Purification of the enzymes*

About 800 g of freeze-dried cells suspended in 50 mM phosphate buffer (pH 6.5) were treated with an MSK Cell Homogenizer (B. Braun Japan Co., Ltd.) for 30 sec. The container was cooled with cold carbon dioxide gas and the treatment was repeated 8 times. The resultant homogenate was centrifuged at 10,000 *xg* for 30 min to obtain cell-free extract. Then, it was fractionated by ammonium sulfate precipitation. The fraction precipitated between 40% and 70% saturation of ammonium sulfate was collected by centrifugation and dissolved in 50 mM glycine-NaOH buffer (pH 9.0). After the insoluble material was removed by centrifugation at 100,000 *xg* for 1 hr, the enzyme solution was dialyzed against the same buffer at 4°C for 24 hr. The dialyzed enzyme solution was put on a DEAE-Toyopearl 650S column (1.4 x 20 cm) equilibrated with 50 mM glycine-NaOH buffer (pH 9.0). Proteins were eluted by a linear 0-100 mM gradient of sodium chloride in the same buffer. The active fractions were collected, concentrated by ammonium sulfate precipitation, and put on an AF-Blue Toyopearl 650ML column (1.4 x 20 cm) equilibrated with 10 mM phosphate buffer (pH 6.0). Proteins were eluted by a linear 0-200 mM gradient of sodium chloride in the same buffer. Although the enzyme was eluted from the column with 50 mM sodium chloride, most of the activities were found in the breakthrough fractions as shown in Fig. III-10(A). These fractions were pooled and rechromatographed on the same column (Fig. III-10(B)). Most of the activities were eluted again at the breakthrough (I) but some activities were adsorbed and eluted from the column with 33 mM sodium chrolide (II).

The first fractions in the rechromatography (I) were further

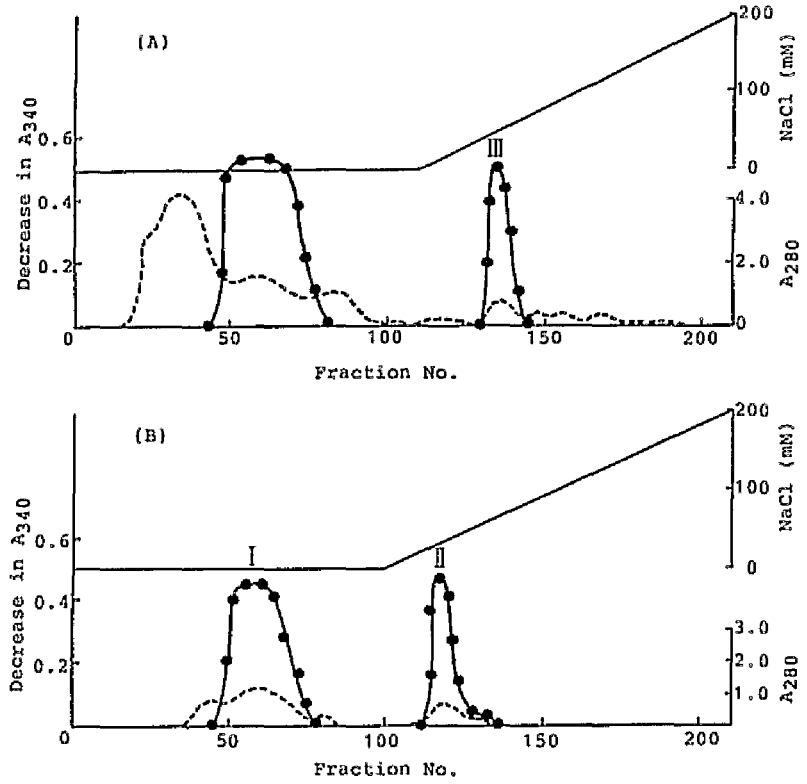


Fig. III-10 Elution profiles of erythrose reductase from AF-Blue Toyopearl 650ML column.

A: First chromatography. B: Rechromatography of breakthrough on first chromatography. ●—●: decrease in absorbance at 340nm, ----: absorbance at 280nm.

subjected to chromatofocusing on a PBE 94 column (1.5 X 20 cm) equilibrated with 25 mM histidine-HCl buffer (pH 6.2). Proteins were eluted with Polybuffer 74 adjusted to pH 4.0. A single sharp activity peak was obtained at pH 5.2. The peak fractions were collected and used as a purified enzyme (ER-1) in the following experiments. The second fractions in the rechromatography (II) and the active fractions eluted from the column in the first chromatography (III) were subjected to hydrophobic chromatography on a Butyl-Toyopearl 650S column (11 X 20 cm) equilibrated with 35% saturated ammonium sulfate in 10 mM phosphate buffer (pH 6.0). Proteins were eluted by a linear gradient of 35 to 20% saturated ammonium sulfate in the same buffer. A single activity peak was eluted with 25% saturated ammonium sulfate in each chromatography. The active fractions were pooled and used as purified enzymes ER-2 and ER-3, respectively.

The results of purification are summarized in Table III-1. The specific activities of ER-1, ER-2, and ER-3 were 140, 150, and 150 U/mg, respectively. More than 500-fold purification was achieved over the cell-free extract.

Table III-1 Purification scheme of erythrose reductase.

| Step | Total activity (units) | Total protein (mg) | Specific activity (units/mg) | Purification (fold) | Recovery (%) |
|---|---|---|---|---|---|
| Cell-free extract | 23000 | 85000 | 0.27 | 1 | 100 |
| Ammonium sulfate (40–70%) saturation | 11000 | 31000 | 0.35 | 1.3 | 48 |
| DEAE-Toyopearl 650S | 5900 | 1700 | 3.5 | 13 | 26 |
| AF-Blue Toyopearl 650ML | | | | | |
| I | 2000 | 200 | 9.9 | 37 | 8.6 |
| II | 790 | 44 | 18 | 68 | 3.5 |
| III | 900 | 43 | 21 | 77 | 3.9 |
| Chromatofocusing of I | | | | | |
| ER-1 | 800 | 5.7 | 140 | 520 | 3.5 |
| Butyl-Toyopearl 650S of II and III | | | | | |
| ER-2 | 270 | 1.8 | 150 | 550 | 1.2 |
| ER-3 | 460 | 3.0 | 150 | 560 | 2.0 |

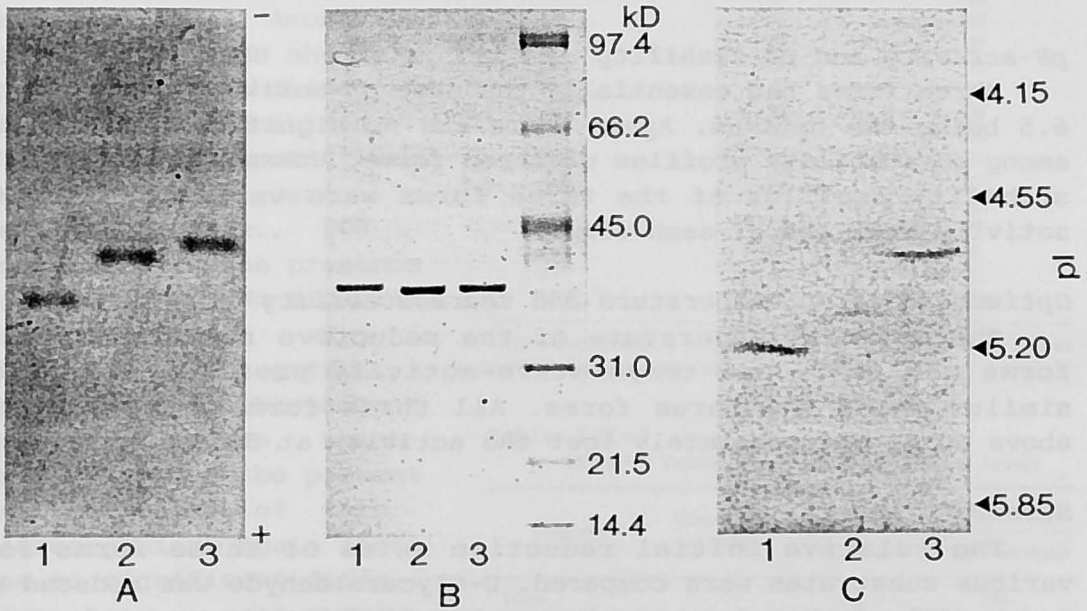


Fig. III-11 Polyacrylamide gel electrophoresis of three purified enzymes.
A: Native PAGE, B: SDS-PAGE, C: IEF-PAGE. 1: ER-1, 2: ER-2, 3: ER-3. For details, see MATERIALS AND METHODS. SDS-PAGE Standards LMW of Bio-Rad Laboratories and *pI* Calibration Kit, pH 2.5–6.5, of Pharmacia LKB Biotechnology were used, respectively, for SDS- and IEF-PAGE.

*Homogeneity of the purified enzymes*

Each purified enzyme showed a single protein band on native PAGE (Fig. III-11(A)), SDS-PAGE (Fig. III-11(B)), and IEF-PAGE (Fig. III-11(C)). In all cases, a single protein band was detected for each sample. In the native PAGE, 2 sets of gels were run and after the electrophoresis one set was stained for erythrose reductase activity and the other for protein. Active

bands completely coincided with respective protein bands (data not shown). These results showed that the purified enzymes were homogeneous.

*Physical properties*

From the data of SDS-PAGE, the molecular weights of the enzymes were calculated to be 38,000 for ER-1 and 37,000 for ER-2 and ER-3. All three forms eluted at the same position on gel filtration using Asahi Pack GS-510. The molecular weight of native enzymes was calculated to be 37,000. Therefore, they have a monomeric structure.

From the data of IEF-PAGE, the isoelectric points (p*I*s) were estimated to be 5.2, 5.0, and 4.8 for ER-1, ER-2, and ER-3, respectively. The p*I* of ER-1 determined by chromatofocusing gave the same value of 5.2.

*pH-activity and pH-stability*

Three forms had essentially the same pH-activity profiles, pH 6.5 being the optimum. Again there was no significant difference among pH-stability profiles of three forms. Unexpectedly, the pH-stability profiles of the three forms were very close to pH-activity profiles of each form.

*Optimum reaction temperature and thermostability*

The optimum temperature of the reductive reaction of all forms was 45°C, and temperature-activity profiles were also similar among the three forms. All three forms were unstable above 40°C, and completely lost the activity at 50°C.

*Substrate specificity*

The relative initial reduction rates of three forms for various substrates were compared. D-Glyceraldehyde was reduced at a rate 65% of that for D-erythrose by all forms. Relative rate of L-erythrulose reduction was about 40% and those of dihydroxyacetone reduction were 36% with ER-1 and about 20% with ER-2 and ER-3. No other aldose and ketose were reduced at an appreciable rate. Thus, substrate specificities of three forms were almost the same.

*Kinetic parameters*

The $K_m$ and $V_{max}$ for D-erythrose of the three forms were determined. ER-1, ER-2, and ER-3 showed similar $K_m$ of 7.1, 7.6, and 8.2 mM and $V_{max}$ of 0.53, 0.38, and 0.63 μmol/min/mg, respectively. The $K_m$ and $V_{max}$ for NADPH of ER-3 were 26 μM and 125 μmol/min/mg, respectively.

*Effects of metal ions and other compounds*

The reductive activity of ER-3 in the presence of metal ions (10 mM) or various compounds was measured (Tables III-2 and III-3). Ten mM of D-erythrose and D-glyceraldehyde were used as substrate solutions. $Ag^{+}$ and $Zn^{2+}$ strongly inhibited the activity (The remaining activity was 20-30%). $Cu^{2+}$, $Fe^{2+}$, $Fe^{3+}$, and $Al^{3+}$ showed less inhibition (The remaining activity was 60-70%). No tested metal ions increased the activity. There is no significant difference between activities to D-erythrose and to D-glyceraldehyde.

EDTA neither inhibited nor activated erythrose reductase activity, as presumed from the results in Table III-2. Among the SH reagents, DTNB showed a significant inhibition while PCMB inhibited slightly. SH protective reagents, too, were inhibitors, i.e., 80% inhibition in the presence of 10 mM DTT. No significant inhibition was observed by sugars, polyols, and phosphate esters of sugars, which are presumed to be present in the cells of this microorganism. Again there was no significant difference between activities to D-erythrose and to D-glyceraldehyde.

Table III-2

Effects of Metal Ions on the Reductive Activity

| Metal ion | Activity (%) to | |
|---|---|---|
| | D-Erythrose | D-Glyceraldehyde |
| None | 100 | 100 |
| $K^{+}$ | 89 | 84 |
| $Na^{+}$ | 91 | 80 |
| $Ag^{+}$ | 23 | 31 |
| $Ca^{2+}$ | 84 | 73 |
| $Mg^{2+}$ | 89 | 84 |
| $Zn^{2+}$ | 37 | 28 |
| $Mn^{2+}$ | 98 | 105 |
| $Ba^{2+}$ | 80 | 84 |
| $Co^{2+}$ | 95 | 77 |
| $Cu^{2+}$ | 67 | 85 |
| $Fe^{2+}$ | 78 | 74 |
| $Fe^{3+}$ | 62 | 48 |
| $Al^{3+}$ | 53 | 72 |
| $MoO_4^{2-}$ | 97 | 93 |

The reductive activity in the presence of 10 mM metal ions was assayed under the standard assay conditions.

Table III-3

Effects of Various Compounds on the Reductive Activity

| Compound | Concentration (mM) | Activity (%) to | |
|---|---|---|---|
| | | D-Erythrose | D-Glyceraldehyde |
| None | | 100 | 100 |
| EDTA | 5 | 92 | 97 |
| DTNB | 1 | 35 | 45 |
| PCMB | 1 | 69 | 75 |
| Mercaptoethanol | 10 | 87 | 84 |
| DTT | 10 | 13 | 19 |
| Glutathione | 10 | 80 | 81 |
| Sodium azide | 10 | 80 | 86 |
| Glucose | 800 | 101 | 84 |
| Erythritol | 800 | 69 | 72 |
| Glycerol | 800 | 73 | 78 |
| Ethanol | 100 | 90 | 99 |
| ATP | 10 | 83 | 96 |
| Glucose-6-P | 10 | 95 | 97 |
| Erythrose-4-P | 10 | 88 | 93 |
| Glyceraldehyde-3-P | 10 | 82 | 87 |

DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoic acid; DTT, dithiothreitol.

Table III-4 Effects of metal ions on the reductive activity.

| Metal ion | Activity (%) to | | | | | |
|---|---|---|---|---|---|---|
| | D-Erythrose | | | D-Glyceraldehyde | | |
| | ER-1 | ER-2 | ER-3 | ER-1 | ER-2 | ER-3 |
| $Ag^{+}$ | 8.7 | 7.2 | 23 | 7.2 | 4.5 | 30 |
| $Fe^{2+}$ | 57 | 100 | 78 | 60 | 100 | 74 |
| $Fe^{3+}$ | 20 | 75 | 63 | 14 | 65 | 50 |
| $Al^{3+}$ | 52 | 66 | 53 | 58 | 88 | 71 |

The reductive activity in the presence of 10mM metal ions was assayed under the standard conditions. Respective activities assayed in the absence of metal ions were taken as 100%.

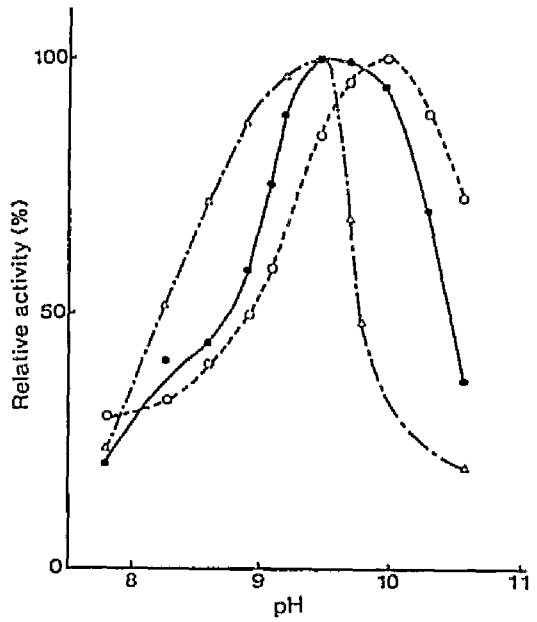


Fig.III-12 Effects of pH on the oxidative activity of three enzymes.
△- - -△: ER-1, O····O: ER-2, ●—●: ER-3. Standard assay conditions for the oxidative activity were used except for boric acid-sodium hydroxide buffer.

Effects of $Ag^{+}$, $Fe^{2+}$, $Fe^{3+}$, and $Al^{3+}$, which showed certain inhibitory effects on ER-3, on the three forms are compared in Table III-4. $Ag^{+}$ exhibited the strongest inhibitory effect and $Fe^{2+}$ the weakest. Among the three forms, ER-1 was more sensitive to these metal ions than the other two. Essentially the same inhibitory patterns were observed when D-glyceraldehyde was used as a substrate instead of D-erythrose.

*Oxidative reaction*

No appreciable activity was observed when the oxidative activity of the three forms was assayed using erythritol at pH

Table III-5 Relative oxidation extent for various substrates.

| Substrate | Relative extent (%) | | |
|---|---|---|---|
| | ER-1 | ER-2 | ER-3 |
| Glycerol | 88 | 150 | 56 |
| Erythritol | 100 | 100 | 100 |
| Arabitol | 55 | 86 | 74 |
| Xylitol | 52 | 72 | 60 |
| Sorbitol | 130 | 340 | 180 |
| Mannitol | 33 | 0 | 0 |

9.5. The oxidative activity of three forms was found only at alkaline conditions after the enzyme was incubated for 10 hr. Fig. III-12 shows pH-activity profiles of the three forms. Obviously, remarkable differences were observed among them. ER-1 and ER-3 showed the maximum activity at pH 9.5 while ER-2 showed the maximum at pH 10.0. Although the obtained rates do not stand for accurate initial rates, it must be pointed out that these activities are much lower compared with the reductive activities of each form; 0.04% with ER-1 and ER-2 and 0.05% with ER-3.

Another unique point of the oxidative activity is that all forms showed broad substrate specificity as summarized in Table III-5. In addition to erythritol and glycerol, both arabitol and xylitol were oxidized at appreciable rates. Furthermore, sorbitol was found to be the most suitable substrate for all three forms. The three forms clearly showed different substrate specificities unlike in the case of the reductive reaction.

## DISCUSSION

At first, it was expected that the erythrose reductase could be separated from glycerol dehydrogenase (acting on glyceraldehyde and dihydroxyacetone). However, after the complete purification, the enzyme still showed both activities. Behaviors of both activities toward metals (Table III-2) and other compounds (Table III-3) also support the conclusion that the same protein catalyzes the production of both erythritol and glycerol. This coincides with the fact that both erythritol and glycerol temporally accumulate in *Aureobasidium* sp. SN-G42 cells.

Many studies have been reported on the enzymes which catalyze the formation of glycerol by oxidation of glyceraldehyde and/or dihydroxyacetone (33, 34). Contrary to this, limited reports are available on the enzymes which catalyze interconversion between erythrose and erythritol. Erythrose reductase of *Schizophyllum commune* (35) and polyol dehydrogenase of silkworm (36) were

Table III-6 Comparison of Substrate Specificity of Several Oxidoreductases

| | Erythrose reductase | Glycerol dehydrogenase[39] (EC 1.1.1.72) | Aldose reductase[37] (EC 1.1.1.21) | Polyol dehydrogenase[38] (EC 1.1.1.21) | Polyol dehydrogenase[36] (EC 1.1.1.21) |
|---|---|---|---|---|---|
| D-Erythrose | 100 | 100 | 100 | 100 | 100 |
| D-Glyceraldehyde | 66 | 135 | 104[a] | 111 | 76 |
| Dihydroxyacetone | 20 | 5 | — | 0 | 4 |
| D-Xylose | 1 | — | 63 | 56 | 15 |
| D-Ribose | 1 | 7 | 59 | 24 | 7 |
| D-Arabinose | 0 | 3 | 34 | 0 | 4 |
| D-Glucose | 0 | <0.5 | 10 | 7 | 0 |

Activity to D-erythrose was taken as 1.00.
[a] Data for DL-glyceraldehyde.

reported to show high erythrose-reducing activities, although their preparations were crude. Besides these, erythrose-reducing activity has been reported with aldose reductase of calf lens (37), polyol dehydrogenase of *Candida utilis* (38), and glycerol dehydrogenase and aldehyde reductase of rabbit skeletal muscle (39). These enzymes showed the highest activity toward glyceraldehyde and reduced D-erythrose at rates from 41 to 96% of those of glyceraldehyde reduction.

The erythrose reductase reported in this section is, therefore, the first example of a purified enzyme that shows the highest activity toward D-erythrose. As shown in Table III-6, this enzyme is specific to the production of erythritol and glycerol under physiological conditions, while other enzymes produced glycerol, xylitol, ribitol, arabitol, or sorbitol, in addition of erythritol. It requires NADPH as a cofactor and its equilibrium strongly favors the reduction of D-erythrose in accordance with all the enzymes described above. It is considered to be present in cytoplasm, like other enzymes.

Three forms of erythrose reductase were purified and characterized from *Aureobasidium* sp. SN-G42. Physicochemical and enzymatic properties of the three forms were compared as summarized in Table III-7. They have approximately the same molecular weight of 37,000 and no subunit structure. They have different pIs and can be clearly separated on IEF-PAGE. The enzymes have high reductive activity and there was no significant difference among their enzymatic properties except for the inhibitory effect of metal ions. The three forms showed remarkable diversity in pH-activity profile and substrate specificity in the oxidative reaction, although their oxidative activity is quite low compared with the reductive one.

It was reported that erythrose reductase of *S. commune* (35), aldose reductase of calf lens (37), and polyol dehydrogenase of *C. utilis* (38) showed $K_m$ for D-erythrose of 5, 0.4, and 0.92 mM,

Table III-7 Comparison of some enzymatic properties of three forms of erythrose reductase.

| | ER-1 | ER-2 | ER-3 |
|---|---|---|---|
| Molecular weight | 38,000 | 37,000 | 37,000 |
| Isoelectric point | 5.2 | 5.0 | 4.8 |
| Reductive reaction | | | |
| Specific activity | 140 U/mg | 150 U/mg | 150 U/mg |
| Optimum pH | 6.5 | 6.5 | 6.5 |
| pH-stability | 6.0–7.0 | 6.0–7.0 | 6.0–7.0 |
| Optimum temp. | 45°C | 45°C | 45°C |
| Thermostability | 40°C | 40°C | 40°C |
| Substrate specificity | ERY>GA>DHA[a] | ERY>GA>DHA | ERY>GA>DHA |
| Inhibition by $Ag^+$ | 8.7% | 7.2% | 23% |
| Inhibition by $Fe^{3+}$ | 20% | 75% | 63% |
| Oxidative reaction | | | |
| Specific activity* | 5.6 U/mg | 6.0 U/mg | 7.5 U/mg |
| Optimum pH | 9.5 | 10.0 | 9.5 |
| Substrate specificity | Sor>Ery>Gly[b] | Sor>Gly>Ery | Sor>Ery>Gly |

* Calculated from the extent of erythritol oxidation after 10 h.

[a] ERY: D-erythrose; GA: D-glyceraldehyde; DHA: dihydroxyacetone.

[b] Sor: sorbitol; Ery: erythritol; Gly: glycerol.

respectively. All forms of erythrose reductase of *Aureobasidium* sp. SN-G42 showed a little higher $K_m$ than that of erythrose reductase of *S. commune*.

The facts that the oxidative reaction of three forms of erythrose reductase is much slower compared with the reductive one and that they are active only at strong alkaline pH (Fig. III-12) indicated that the differences in pH-activity profile and substrate specificity in the oxidative reaction have no practical meaning in the living cells, where the pH is weakly acidic. Therefore, this enzyme, with all three forms, is supposed to catalyze exclusively the reduction of D-erythrose (formation of erythritol) in *Aureobasidium* sp. SN-G42 cells.

As described in III-1, it is supposed that intracellular erythritol in *Aureobasidium* sp. SN-G42 could contribute to its osmoregulation in the presence of a high concentration of sugars. Erythrose reductase is, therefore, critically important to this microorganism and this may be one of the reasons for the presence of multiple forms.

## III-3. Lipid composition of cell membrane

Cell membranes are believed to be composed of lipid bilayers in which proteins float. It is thought that the proteins in membranes can move by lateral diffusion because lipid bilayers have fluidity. This "Fluid Mosaic Model", proposed by Singer and

Nicolson (40), indicates that many functions of the membrane appear through its fluidity. The main components relating to the fluidity of membranes are lipids, in particular, phospholipids and sterols.

This section describes the contents and compositions of phospholipids and sterols in some sugar-tolerant yeasts.

## MATERIALS AND METHODS

*Strains.* Seven yeast strains, *D. hansenii* 67-40A, *H. anomala* 150-40A, *T. delbrueckii* 154-CA, *Z. rouxii* 88-40A and 151-40A, *C. tropicalis* 118-40A, and *Aureobasidium* sp. SN-G42, were examined.

*Extraction and fractionation of lipids.* Cells grown in media containing 1% or 45% (w/w) glucose were harvested in the late phase of logarithmic stage. Lipids were extracted from 0.2 g of freeze-dried cells using a solvent consisting of chloroform and methanol (1:1 (v/v)). The extracts were dried up and supplemented with 50 ml of a mixture of chloroform and methanol (2:1 (v/v)) and 10 ml of 0.9% NaCl. Chloroform layer, obtained by shaking and standing the solution, was completely dried up, and weighed.

The extracted lipids were dissolved in a small amount of chloroform and put on a column (0.8 x 10 cm) packed with 2 g silica gel (Silica gel 60, 0.063-0.200 mm, 70-230 mesh, MERCK). Lipids were fractionated by eluting with 40 ml of chloroform, 160 ml of acetone, and 40 ml of methanol at a rate of 1 ml/min. Two fractions eluted with chloroform and methanol were used as sample solutions containing sterols and phospholipids, respectively, for the following experiments.

*Determination of phospholipids and ergosterol.* Phospholipids and ergosterol were determined by colorimetry.

Phospholipid content. Sample solutions were dried up by flushing nitrogen gas and 0.5 ml perchloronic acid was added. $KH_2PO_4$ solutions containing 1-5 μg phosphorus were supplemented with 0.5 ml perchloronic acid and heated at 100°C to evaporate water. The sample and standard solutions were heated at 180°C for 7 hr and cooled to room temperature, followed by adding 4.6 ml of ammonium moribdate and 0.2 ml of Fiske-Subbarow reagent and boiling for 7 min. After cooling, absorbance at 830 nm was measured.

Ergosterol content. Sample solutions and standard solutions containing 0.1-0.5 mg ergosterol were dried up using nitrogen gas and dissolved in 0.5 ml acetic acid. The solutions were supplemented with 1 ml of a mixture of acetic acid anhydride and sulfonic acid (19:1) and mixed well. After standing for 30 min, absorbance at 660 nm was measured.

*Analysis of phospholipid composition.* The composition of phospholipids was determined by thin layer chromatography. Sample solutions containing about 0.6 mg phospholipids and authentic phospholipids were put on Uniplate, Silica gel plates (ANATECH Co.) and developed with $CHCl_3$ - MeOH - AcOH - $H_2O$ (85:20:10:3.5). Spots of phospholipids were detected using $I_2$ vapor and a ninhidrin reagent and taken off from plates. The amount of phospholipids in each spot was measured by the method described above.

*Analysis of fatty acid composition of phospholipids.* About 20 mg of dried cells and 2 ml of 5% HCl - MeOH were put into test tubes and flushed with nitrogen gas. They were heated at 100°C for 3 hr and cooled. Methylated fatty acids were extracted by adding 0.5 ml water and 1.5 ml hexane and taking hexane layer. Extraction was repeated 4 times. The hexane layer was collected, washed with 6 ml of water, and dehydrated with anhydrous sodium sulfate. The extract was concentrated by flushing nitrogen gas and its fatty acid composition was analyzed using a gas chromatograph (Shimadzu GC-14A) equipped with a DEGS (15%, 80/100 mesh) column.

## RESULTS

The primary sterol in tested strains was identified as ergosterol by gas chromatography. The contents of phospholipids and ergosterol in seven strains were shown in Table III-8. Phospholipid content of *D. hansenii* 67-40A cells grown in 45%

Table III-8 Phospholipid and free sterol content of some strains of sugar-tolerant yeasts grown at different concentrations of glucose

| Strain | Glucose (%, w/w) | Phospholipid (ug/mg dry wt. cells) | Ergosterol (ug/mg dry wt. cells) |
|---|---|---|---|
| Debaryomyces hansenii 67-40A | 1 | 22.4 | 7.2 |
| | 45 | 37.6 | 8.0 |
| Hansenula anomala 150-40A | 1 | 31.0 | 18.8 |
| | 45 | 14.8 | 16.8 |
| Torulaspora delbrueckii 154-CA | 1 | 31.7 | 22.4 |
| | 45 | 20.2 | 32.0 |
| Zygosaccharomyces rouxii 88-40A | 1 | 30.4 | 11.5 |
| | 45 | 22.2 | 10.2 |
| Zygosaccharomyces rouxii 151-40A | 1 | 19.5 | 9.0 |
| | 45 | 18.2 | 12.6 |
| Candida tropicalis 118-40A | 1 | 28.6 | 3.8 |
| | 45 | 31.5 | 7.3 |
| Aureobasidium sp. G42 | 1 | 19.4 | 4.7 |
| | 45 | 19.0 | 11.1 |

Table IV-9 Phospholipid composition of Zygosaccharomyces rouxii and Aureobasidium sp. grown at different concentrations of glucose

| Strain | Glucose (%, w/w) | Phospholipid composition (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | PC | PE | PI | PS | PA | CL |
| Z. rouxii 88-40A | 1 | 49.4 | 13.9 | 18.2 | 8.6 | 9.9 | - |
| | 45 | 48.2 | 13.5 | 11.2 | 6.6 | 20.5 | - |
| Z. rouxii 151-40A | 1 | 58.1 | 13.6 | 11.9 | 9.3 | 7.1 | - |
| | 45 | 53.1 | 15.4 | 10.9 | 11.7 | 8.9 | - |
| Aureobasidium sp. G42 | 1 | 44.9 | 21.5 | 19.5 | 5.7 | - | 8.4 |
| | 45 | 39.9 | 19.2 | 17.0 | 13.3 | - | 10.6 |

Table IV-10 Fatty acid composition of phospholipids of Zygosaccharomyces rouxii and Aureobasidium sp. grown at different concentrations of glucose

| Strain | Glucose (%, w/w) | % of fatty acid | | | | | | U.I. |
|---|---|---|---|---|---|---|---|---|
| | | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 | |
| Z. rouxii 88-40A | 1 | 18.5 | 6.4 | 8.0 | 39.3 | 27.8 | - | 101.3 |
| | 45 | 16.4 | 4.0 | 4.8 | 58.9 | 15.9 | - | 94.7 |
| Aureobasidium sp. G42 | 1 | 21.9 | 0.4 | 2.0 | 45.5 | 22.5 | 7.7 | 114.0 |
| | 45 | 19.8 | 0.5 | 1.7 | 42.8 | 32.5 | 2.7 | 113.7 |

glucose-medium was higher than that in 1% glucose-medium. On the other hand, those of *H. anomala* 150-40A, *T. delbrueckii* 154-CA, and *Z. rouxii* 88-40A were lower in 45% glucose-medium than in 1% glucose-medium. Phospholipid contents of other three strains were not affected by glucose concentration of cultivation media. Ergosterol contents of *T. delbrueckii* 154-CA, *C. tropicalis* 118-40A, and *Aureobasidium* sp. SN-G42 were higher in 45% glucose-medium than in 1% glucose-medium, while those of other strains were almost constant.

Table III-9 shows phospholipid compositions of three extremely sugar-tolerant strains. In *Z. rouxii* 88-40A, the content (%) of phosphatidyl inositol (PI) was lower and that of phosphatidic acid (PA) was higher in 45% glucose-medium than in 1% glucose-medium. In the case of *Z. rouxii* 151-40A and *Aureobasidium* sp. SN-G42, the contents (%) of phosphatidyl serine (PS) were higher and those of phosphatidyl choline (PC) were lower in 45% glucose than in 1% glucose.

Fatty acid compositions of phospholipids in *Z. rouxii* 88-40A and *Aureobasidium* sp. SN-G42 were determined as shown in Table

III-10. In *Z. rouxii* 88-40A cells grown in 45% glucose-medium, the percentage of oleic acid was higher and that of linoleic acid was lower, resulting in a lower unsaturated index (U. I.) compared with those in 1% glucose-medium. On the other hand, in *Aureobasidium* sp. SN-G42, U. I. in the presence of different concentrations of glucose were almost constant although some differences were observed in fatty acid composition.

## DISCUSSION

It was suggested that cell membranes of salt-tolerant yeasts may play an important role in maintaining a low internal sodium ion concentration in high-salt media, based on the observations by Onishi (41). Content and composition of phospholipids and sterols, primary lipid components of cell membranes, may closely relate to the structures and functions of cell membranes. However, the results in the previous studies on phospholipids of salt-tolerant yeasts did not always agree with each other (16-19).

Fatty acid composition of membrane lipids is also important as one of factors determining the functions of cell membranes. Previously, general decreases in the extent of unsaturation of total fatty acids, caused by increases in oleic acid and decreases in linoleic acid, at increased salinity were recognized in *Z. rouxii* (15, 17, 19). On the other hand, only minor changes in fatty acid composition were reported for *D. hansenii* grown at various concentrations of sodium chloride (18).

The results in this study indicated that the effects of sugar concentration of media on the lipid composition of cell membranes of sugar-tolerant yeasts varied with species and strains. Therefore, it is concluded that the lipid composition of cell membranes may not always relate to sugar-tolerance of yeasts.

### III-4. $H^+$-pumping activity across cell membrane

Cell membrane bound $H^+$-ATPase (42) is called a proton pump. Proton gradient across membranes is produced by the energy generated in the ATP hydolysis by this enzyme, and directly and indirectly cause the active transport of amino acids, sugars, and organic acids. Oxidoreductases including NAD(P)H-ferricyanide oxidoreductase are known as other enzymes acting $H^+$-pumping (43). The strength of energy metabolism in cell membranes of sugar-tolerant yeasts was investigated using the activities of these enzymes as indicators.

## MATERIALS AND METHODS

*Strains.* Three strains, *S. cerevisiae* 89-25A, *Z. rouxii* 88-40A, and *Aureobasidium* sp. SN-G42 were used.

*Isolation and purification of cell membrane fraction from yeast strains.* Cells grown in media containing 1% or 45% (w/w) glucose were harvested in the late phase of logarithmic stage. Cell membrane fractions were isolated and purified as shown in Fig. III-13.

*Assay of $H^+$-ATPase activity.* Each sample was incubated at 38 $^oC$ for 30 min in 500 µl of reaction mixture containing 3 mM $MgSO_4$, 3 mM ATP-Tris, 50 mM KCl, 30 mM MES-Tris (pH 6.5), 1 mM $NaN_3$, 1 mM $NH_4Mo$, 0.025% Triton X-100, and 30 µg cell membrane vesicles. The reaction was terminated with 100 µl of 50% trichloroacetic acid. Phosphorus liberated from ATP in this reaction was determined by Takahashi method (44).

*Assay of NADH-ferricyanide oxidoreductase activity.* NADH-ferricyanide oxidoreductase activity was assayed in 1 ml of 10 mM Tris-Mes (pH 7.5), 0.8 mM $K_3Fe(CN)_6$ and 200 µl (60 µg) of the membrane fraction. The reaction was initiated by adding NADH to a final concentration of 0.1 mM. The loss of absorption was measured at 340 nm, using the identical solution without membranes as a blank.

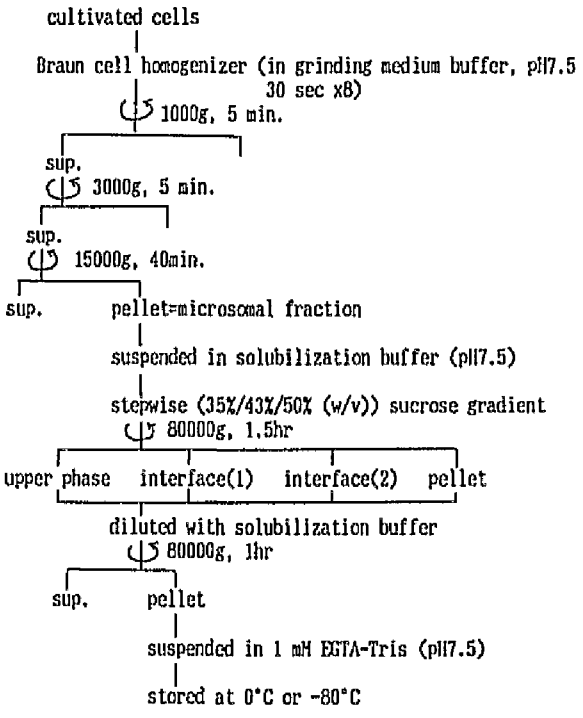


Fig.III-13 Method for isolation and purification of plasma membrane fraction from yeast cells

Table III-11
Comparison of plasma membrane $H^+$-ATPase activity

| Strain | Specific activity (μmoles $P_i$/min/mg protein) | |
|---|---|---|
| | 1% glucose-medium | 45%(w/w) glucose-medium |
| S. cerevisiae 89-25A | 1.357 | 0.339 |
| Zygosaccharomyces rouxii 38-40A | 0 | 0 |
| Aureobasidium sp. G42 | 1.120 | 1.190 |

## RESULTS

$H^+$-ATPase activities in cell membranes of three strains were shown in Table III-11. The activity of *S. cerevisiae* 89-25A grown in 45% glucose-medium was much lower than that in 1% glucose-medium, while that of *Aureobasidium* sp. SN-G42 grown in 45% glucose was as high as that in 1% glucose. These activities were found to be correlated with $H^+$-pumping activity of each $H^+$-ATPase. On the other hand, no activity of this enzyme was detected in *Z. rouxii* 88-40A.

*Z. rouxii* 88-40A showed NADH-ferricyanide oxidoreductase activities in both media. However, the activities were less than half of those of *Aureobasidium* sp. SN-G42 (data not shown).

## DISCUSSION

Ions and metabolic constituents are actively transported across cell membranes. $Na^+/K^+$-activated ATPase, which could serve as a sodium pump, is supposed to be particularly important for maintaining a low internal sodium ion concentration in cytosol of salt-tolerant yeasts under a high salt environment. Steinkraus et al. (23) and Ayres et al. (24) suggested that this enzyme played a role in adaptation of *Z. rouxii* cells to a high salt environment.

Sodium ions may be transported by a sodium-proton antiporter generated by proton gradient across membranes (45). The proton gradient is formed by $H^+$-ATPase. Watanabe et al. (25) found that there were slight differences in the enzymatic properties of cell membrane $H^+$-ATPase between *Z. rouxii* and *S. cerevisiae* and that specific activity of the enzyme from *Z. rouxii* was higher in cells grown in the presence of a high concentration of salt than that in the absence of salt. In the present study, cell membrane $H^+$-ATPase activity of a yeast species with low tolerance to sugar (*S. cerevisiae*) was depressed in a high concentration of glucose,

while the activity of a species with high tolerance (*Aureobasidium* sp.) was not depressed. These results suggested that $H^+$-ATPase activity might relate to sugar-tolerance. However, in the case of *Z. rouxii*, representative sugar-tolerant yeast, the activity was not noted, thus, the activity was not always a factor for determining sugar-tolerance.

## III-5. Membrane fluidity and microenvironmental characterization

Thermal motion of membrane components, commonly termed "membrane fluidity", is now believed to play an important role in cellular regulatory mechanisms. In the present study, the microviscosity of the surface membrane lipid layer was determined with the aid of a fluorescent hydrocarbon probe.

The spectral properties of a solubilized optical probe such as pyrene provide some important information on intermolecular interactions between the probe and the surrounding lipid layers. The micropolarity in the close vicinity of the probe molecule (microenvironment) can be evaluated by analysis of the spectral properties of the probe.

### MATERIALS AND METHODS

*Strains*. Three strains used in III-4 were examined.

*Isolation and purification of cell membrane fraction*. The three strains were grown in media containing 1% or 45% (w/w) glucose. *Aureobasidium* sp. SN-G42 cells were harvested at a constant interval and the other strains in the middle phase of logarithmic stage. Cell membrane fractions were isolated and purified as described in III-4.

*Measurement of temperature dependence of membrane fluidity*. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was solubilized into isolated cell membranes by incubating at $0^{\circ}C$ for overnight after the addition of saturated DPH solution. In general, the fluorescence emitted molecules which are dispersed in a viscous medium such as membrane lipid layers is partially polarized. This is ustomarily expressed in terms of molecular anisotropy or degree of polarization which relate to the membrane fluidity. The membrane fluidity was measured with fluorescence depolarization method at 5 to $50^{\circ}C$ as described by Sano (46). The activation energy of membrane fluidity ($\Delta E$) was calculated from fluorescence depolarization (P) measured at various temperatures in the following equation.

$$Y = \ln[(3-P)r_0/2P-1]$$
$$= \ln[T_r C(r)/A] - \Delta E/RT$$

where the first term is a constant characterized by the fluorescence probe, R is gas constant, and T is absolute temperature.

*Microenvironment of membrane*. Pyrene was solubilized into cell membranes by incubating at 0°C for overnight as similar to DPH mentioned above. The fluorescence spectra of the pyrene were recorded on a Shimadzu RF-503A difference spectrofluorophotometer at the excitation wavelength of 335 nm. Both excitation and emission slit widths were 10 nm. All measurements were carried out at 25°C. Two fluorescence intensity ratios, $I_{384}/I_{376}$ and $I_{394}/I_{376}$ can be used to monitor microenvironments in the close vicinity of pyrene molecules. The micropolarity of the cell membrane fractions were obtained.

## RESULTS

In the cell membrane of *Aureobasidium* sp. SN-G42 cells, the activation energy of membrane fluidity ($\Delta E$) and the dielectric constant ( $\varepsilon$ ) were almost constant till the middle phase of logarithmic stage in both media. And then, both $\Delta E$ and $\varepsilon$ rapidly increased, showing that the membrane fluidity increased. Therefore, $\Delta E$ and $\varepsilon$ of cell membranes of the three strains were compared in the middle phase of logarithmic stage (Table III-12). There was a difference in $\Delta E$ of cell membranes between *S. cerevisiae* 89-25A cells grown in 45% glucose-medium and in 1% glucose-medium, while no significant difference was observed in other two strains. No significant effect of glucose concentration of media on $\varepsilon$ of cell membranes was observed in all the strains.

Table III-12 Comparison of activation energy of membrane fluidity and dielectric constant of plasma membrane fragments of yeasts grown at different concentrations of glucose

| Strain | Glucose (%, w/w) | Activation energy of membrane fluidity $\Delta E$ (kcal/mole) | Dielectric constant $\varepsilon$ |
|---|---|---|---|
| S. cerevisiae 89-25A | 1 | 1.39 | 14.0 |
| | 40 | 0.94 | 14.0 |
| Z. rouxii 38-40A | 10 | 1.19 | 22.8 |
| | 45 | 1.05 | 24.2 |
| Aureobasidium sp. G42 | 1 | 0.60 | 29.6 |
| | 45 | 0.54 | 29.8 |

## DISCUSSION

Previously, no detailed physiochemical study about the membrane fluidity and rigidity was done on yeasts. Unexpectedly, in the present study, there was no significant effect of glucose concentration of media on fluidity and rigidity of cell membranes. Therefore, sugar-tolerance of the tested yeasts might not relate to the fluidity and rigidity of their cell membranes.

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# CHAPTER IV

## GROWTH CONTROL OF SUGAR-TOLERANT YEASTS

### IV-1. Effect of surfactants

Surfactants are characterized by containing hydrophilic (polar) and hydrophobic (hydrocarbon or nonpolar) group. In the review on structure-function relationships of various classes of surfactants as antimicrobial agents, cationic surfactants were shown to be more active than anionic and nonionic agents (1). In particular, quaternary ammonium salts are widely used because of their wide antibacterial activity spectrum and high antibacterial activity (2, 3). Monoglycerides and sucrose fatty acid esters, used mainly as food emulsifiers, also show antibacterial activity (4-6). For example, sucrose monopalmitate and sucrose monostearate are used for preventing the spoilage of such foods as canned milk coffee. Cholate, a primary component of bile, is reported to increase antibacterial activity of polyphosphates and sodium chloride (7). Although the mechanism of antibacterial action of surfactants has not been completely clarified, surfactants are thought to adsorb onto cells of microorganisms, permeate into cell envelopes, and act on them.

This section describes the effects of some surfactants on the growth of sugar-tolerant yeasts at different $a_w$.

#### MATERIALS AND METHODS

*Strains*. Thirty-three yeast strains belonging to 29 species of 14 genera, isolated from high-sugar foods, were used.

*Surfactants*. Five kinds of non-ionic surfactants with polyoxyethylene (POE), POE (47) laurylether (HLB (Hydrophilic Lipophilic Balance) 16.3), POE (85) nonylphenylether (HLB 18.9), POE monolaurate (13.7), POE (20) sorbitan monolaurate (HLB 16.7), POE (20) sorbitan monoooleate (HLB 11.1) were obtained from Kao corporation. Sucrose fatty acid esters S-1 and S-2 were obtained from Mitsubishi Chemical Industries Ltd. and S-3 from Daiichi Chemical Co., Ltd. Their properties were shown in Table IV-1. Sodium cholate was obtained from Wako Pure Chemicals Co., Ltd.

*Preparation of media*. Media containing 1, 20, or 40% (w/w) glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and surfactants were prepared. Concentrations of sodium cholate were 0.05, 0.10, 0.25, 0.50, and 1.0% and those of sucrose fatty acid ester S-1 were 0.01, 0.02, 0.05, and 0.1%. For the other surfactants, only one concentration of 0.1% was used.

Table IV-1 Sucrose fatty acid esters examined

| Sample name | fatty acid | HLB* | Composition of ester (%) | |
|---|---|---|---|---|
| | | | mono | di, tri, or poly |
| S-1 | stearic acid (70%) | 15 | 70 | 30 |
| S-2 | myristic acid<br>palmitic acid<br>stearic acid | 9.5 | 45 | 55 |
| S-3 | myristic acid<br>palmitic acid<br>stearic acid | 15 | 70 | 30 |

* HLB : Hydrophilic Lipophilic Balance

*Incubation*. Yeast cells preincubated on YM agar were inoculated into tested media ($10^3$ cells/ml) and incubated at 25°C. Incubation periods were 10 days in media containing 1% glucose, 14 days in media containing 20 %(w/w) glucose, and 24 days in media containing 40 %(w/w) glucose. The growth of yeasts was observed during incubation and "relative growth rate" was obtained in comparison with that in a medium not containing surfactants (control). "Relative growth rates" of 0-5 were defined as follows respectively; 0: no growth, 1: slight growth in final stage of incubation, 2: beginning of growth in middle stage of incubation, 3: lower growth rate compared with control, 4: longer induction period compared with control, 5: slightly lower growth rate compared with control. "Growth inhibition index" was defined as the reverse of "relative growth rate".

## RESULTS AND DISCUSSION

Figs IV-1 and IV-2 show the relationships between the concentration of sodium cholate and sucrose fatty acid ester S-1 and their inhibitory effects on the growth of yeasts. In 1% glucose-medium, even the highest concentration (1%) of sodium cholate was not so effective, although the growth of sensitive strains, *S. pombe* 57-50B and *Rhodosporidium* sp. 65-25A was completely inhibited. In 40% glucose, the antimicrobial effect of sodium cholate increased with the increase in its concentration. The concentration dependence of the antimicrobial effect of sucrose fatty acid ester S-1 was lower than that of sodium cholate. The average growth levels of all tested yeasts were relatively high even in media containing 0.1% S-1. The growth of sensitive strains, *C. nodaensis* 24-60A and *C. bombi* 19-25A was significantly depressed in 1% glucose and completely inhibited in

Table IV-2 Effects of 9 surfactants

| Strain | | 1% glucose medium | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SC | S-1 | S-2 | S-3 | E47 | E85 | L12 | T20 | T80 | Total |
| *Debaryomyces hansenii* | 67-40 A | 0.5 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 4.5 |
| *Hansenula anomala* | 150-40 A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Pichia ohmeri* | 19-40 A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Pichia membranaefaciens* | 114-CA | 0 | 4 | 0 | 2 | 4 | 3 | 3 | 3 | 3 | 22 |
| *Saccharomyces cerevisiae* | 89-25 A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Saccharomyces cerevisiae* | 4512 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| *Schizosaccharomyces pombe* | 57-40 B | 0.5 | 4 | 0 | 3 | 0.5 | 0 | 2 | 0 | 0 | 10 |
| *Torulaspora globosa* | 26-40 A | 0.5 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.5 |
| *Torulaspora delbrueckii* | 154-CA | 0.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.5 |
| *Zygosaccharomyces rouxii* | 38-40 A | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Zygosaccharomyces rouxii* | 88-40 A | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 4 |
| *Zygosaccharomyces rouxii* | 151-40 A | 0 | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3 |
| *Zygosaccharomyces rouxii* | 86-A | 0 | 2 | 1 | 1.5 | 0 | 0 | 0 | 1 | 1 | 6.5 |
| *Rhodosporidium* sp. | 65-25 A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Candida apicola* | 22-40 A | 0 | 3 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 6 |
| *Candida bombi* | 19-25 A | 2 | 4 | 1 | 3 | 1 | 1 | 3 | 1 | 0 | 16 |
| *Candida bombicola* | 34-40 B | 1 | 3 | 1 | 2 | 0 | 0 | 1 | 1 | 0 | 9 |
| *Candida dattila* | 128-25 A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Candida dulciaminis* | 155-CA | 2 | 2 | 0 | 1 | 2 | 1.5 | 3 | 2 | 1 | 14.5 |
| *Candida famata* | 147-40 A | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 |
| *Candida glucosophila* | 29-25 A | —* | — | — | — | — | — | — | — | — | — |
| *Candida guilliermondii* | 22-25 A | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 2 |
| *Candida intermedia* | 147-CA | 0 | 0 | 0 | 0.5 | 0.5 | 0 | 0 | 0 | 0 | 1 |
| *Candida lactiscondensi* | 91-40 A | 0 | 1 | 0.5 | 0.5 | 0 | 1 | 3 | 0 | 0 | 6 |
| *Candida mannitofaciens* | 152-40 A | 0 | 4 | 1 | 4 | 0 | 0 | 2 | 2 | 0 | 13 |
| *Candida nodaensis* | 24-60 A | 0 | 4 | 1 | 3 | 1 | 1 | 2 | 1 | 0 | 13 |
| *Candida tropicalis* | 118-40 A | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.5 | 8.5 |
| *Candida versatilis* | 24-60 B | 0 | 5 | 0 | 4 | 0.5 | 0 | 1 | 0.5 | 0 | 11 |
| *Cryptococcus laurentii* | 10-25 A | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 3 | 2 | 7 |
| *Kloeckera apis* | 26-50 A | 0 | 1 | 0 | 0.5 | 2 | 0 | 1 | 1 | 1 | 6.5 |
| *Rhodotorula rubra* | 70-25 A | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 3 | 0 | 6 |
| *Sympodiomycopsis paphiopedili* | 101-40 A | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 5 |
| *Aureobasidium* sp. | G-42 | 0 | 1 | 0 | 1 | 2 | 1 | 2 | 1 | 0.5 | 8.5 |

* no growth

SC: sodium cholate, S-1, S-2, & S-3: sucrose fatty acid ester, E47: POE (47) lauryl ether, E85: T80: POE (20) sorbitan monooleate

Growth inhibition index 0: no inhibition, 1: slightly lower growth rate compared with control, 2: growth in final stage of incubation, 5: no growth

on the growth of 33 yeast strains

Growth inhibition index

| 20% (w/w) glucose medium | | | | | | | | | | 40% (w/w) glucose medium | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SC | S-1 | S-2 | S-3 | E47 | E85 | L12 | T20 | T80 | Total | SC | S-1 | S-2 | S-3 | E47 | E85 | L12 | T20 | T80 | Total |
| 1 | 0 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 9 | 1.5 | 1 | 0 | 2.5 | 3 | 1.5 | 2 | 1 | 0 | 12.5 |
| 1 | 2 | 1 | 2 | 2 | 1 | 0 | 1 | 0 | 10 | 0 | 3 | 0 | 2 | 5 | 1.5 | 5 | 2 | 1 | 19.5 |
| 1 | 2 | 1 | 2 | 0 | 1 | 1 | 0 | 0 | 8 | 0 | 2 | 1 | 2 | 4 | 0 | 2 | 3 | 2 | 16.0 |
| 0 | 4.5 | 3 | 4 | 4.5 | 4 | 3 | 3 | 4 | 30 | 2 | 5 | 3 | 5 | 5 | 5 | 5 | 1 | 1 | 32.0 |
| 2 | 3 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 7 | 4 | 5 | 0 | 4 | 5 | 1 | 2 | 1 | 0 | 22.0 |
| 2 | 4 | 1 | 3 | 0 | 0 | 1 | 0 | 0 | 11 | 5 | 5 | 2 | 5 | 5 | 1.5 | 5 | 1.5 | 0 | 30.0 |
| 2 | 5 | 1 | 4.5 | 3 | 0 | 2.5 | 1 | 0 | 19 | 5 | 5 | 1.5 | 5 | 5 | 0.5 | 3 | 1 | 0.5 | 26.5 |
| 2 | 3 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 8 | 4 | 5 | 1 | 3 | 4 | 0 | 1.5 | 1 | 0 | 19.5 |
| 2 | 2.5 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 7.5 | 5 | 4.5 | 1 | 3 | 4 | 1 | 2 | 1 | 0.5 | 22.0 |
| 0 | 2 | 0.5 | 1 | 0 | 0 | 0 | 0 | 0 | 3.5 | 1 | 3 | 0 | 1 | 5 | 0 | 0 | 0 | 0 | 10.0 |
| 1 | 2 | 0 | 1.5 | 0 | 0 | 1 | 0 | 0 | 5.5 | 1 | 3 | 0 | 1.5 | 3 | 0 | 1 | 1 | 1 | 11.5 |
| 1 | 3 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 7 | 3 | 2.5 | 0 | 2 | 4 | 1 | 2 | 1 | 1 | 16.5 |
| 1 | 3 | 1 | 2 | 1 | 0.5 | 1 | 0 | 0 | 9.5 | 3 | 5 | 2 | 3 | 3 | 1 | 2 | 1 | 1 | 21.0 |
| 2 | 2 | 0 | 1.5 | 1 | 0 | 0 | 0 | 0 | 6.5 | 1 | 3 | 0 | 1.5 | 2.5 | 2 | 0 | 1 | 1 | 12.0 |
| 1.5 | 4.5 | 1 | 4 | 1 | 1 | 3 | 1 | 1 | 18 | 1 | 5 | 0 | 5 | 5 | 0 | 3 | 1 | 0 | 22.0 |
| 0 | 4.5 | 0 | 4 | 0 | 0 | 2 | 1 | 0 | 11.5 | 1 | 5 | 0 | 5 | 4.5 | 1 | 3 | 2 | 0 | 21.5 |
| 0 | 3 | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 9 | 0.5 | 5 | 2 | 5 | 5 | 2.5 | 3.5 | 4 | 0 | 27.5 |
| 2 | 3 | 1 | 2.5 | 1 | 0 | 1 | 0 | 0 | 10.5 | 4 | 4.5 | 1 | 3 | 4 | 1 | 3 | 1 | 1 | 22.5 |
| 2 | 0 | 1 | 1.5 | 2 | 2 | 1.5 | 2 | 1.5 | 13.5 | 5 | 4 | 0 | 1 | 4 | 1 | 5 | 3.5 | 1 | 24.5 |
| 1 | 3 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 10.0 | 5 | 5 | 0 | 4.5 | 5 | 1 | 5 | 1 | 1 | 27.5 |
| 1 | 5 | 2 | 3 | 1 | 1 | 0 | 1 | 0 | 14.0 | 1 | 5 | 3 | 4 | 2 | 1 | 2 | 0 | 0 | 18.0 |
| 1 | 0 | 0 | 1 | 1 | 2 | 1 | 1 | 1 | 8.0 | 4 | 2 | 0 | 2 | 3 | 2 | 2.5 | 2 | 1 | 18.5 |
| 1 | 2 | 1 | 1.5 | 1 | 1 | 1 | 1 | 1 | 10.5 | 4 | 4 | 1.5 | 4 | 5 | 2 | 3 | 3 | 2 | 28.5 |
| 1 | 3 | 2 | 3 | 4 | 1 | 5 | 0 | 0 | 19.0 | 1 | 5 | 1 | 5 | 5 | 1.5 | 5 | 1 | 0 | 24.5 |
| 0 | 4 | 0 | 5 | 3 | 0 | 2 | 2 | 0 | 16.0 | 1 | 5 | 2 | 5 | 5 | 2 | 4.5 | 4 | 1.5 | 30.0 |
| 0 | 5 | 1 | 4 | 2 | 0 | 1 | 1 | 0 | 14.0 | 2.5 | 5 | 2 | 5 | 5 | 3 | 5 | 4 | 2.5 | 34.0 |
| 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2.0 | 2 | 3 | 1 | 3 | 3 | 1.5 | 2 | 3 | 1.5 | 20.0 |
| 0 | 5 | 0 | 5 | 4 | 0 | 1 | 1 | 0 | 16.0 | 1 | 5 | 0 | 5 | 5 | 0 | 3 | 3 | 0 | 22.0 |
| 1 | 0 | 1 | 1 | 4 | 2 | 2 | 2 | 2 | 15.0 | 5 | 4 | 2 | 3 | 5 | 2 | 5 | 3 | 2 | 31.0 |
| 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 3.0 | 5 | 3.5 | 1 | 1 | 5 | 2.5 | 4 | 2.5 | 2 | 26.5 |
| 5 | 0 | 2 | 1 | 3 | 2 | 3 | 2.5 | 2 | 20.5 | 5 | 5 | 2.5 | 4 | 5 | 2.5 | 4 | 3 | 2 | 33.0 |
| 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 7.0 | 0 | 2 | 0 | 1 | 4 | 1 | 1 | 1 | 1 | 11.0 |
| 3 | 2.5 | 0 | 3 | 4 | 1.5 | 2.5 | 2.5 | 2 | 21.0 | 3 | 3 | 0 | 2 | 5 | 1.5 | 4 | 1 | 1 | 20.5 |

POE (85) nonylphenyl ether, L12: POE (12) monolaurate, T20: POE (20) sorbitan monolaurate, lower growth rate compared with control, 3: longer induction period compared with "2", 4: slight

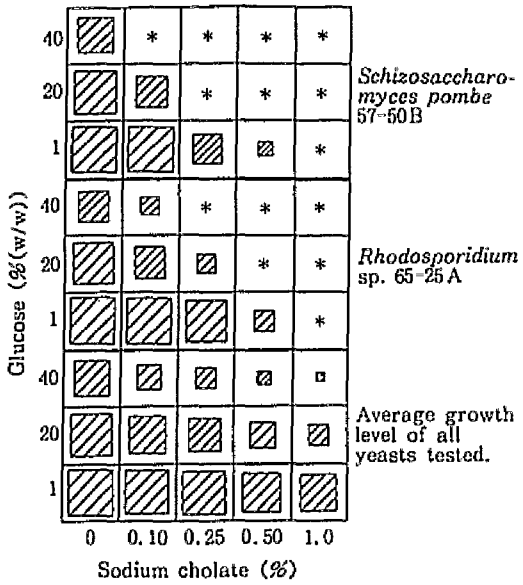


Fig.IV-1 Effects of concentrations of sodium cholate on its inhibitory effects on the growth of yeasts

The area of ▨ shows the growth level of yeasts under different concentrations of sodium cholate. "*" shows "no growth". Growth levels of *Schizosaccharomyces pombe* 57-50 B and *Rhodosporidim* sp. 65-25 A, the growth of which was strongly inhibited by sodium cholate, and average growth level of all yeasts tested were shown.

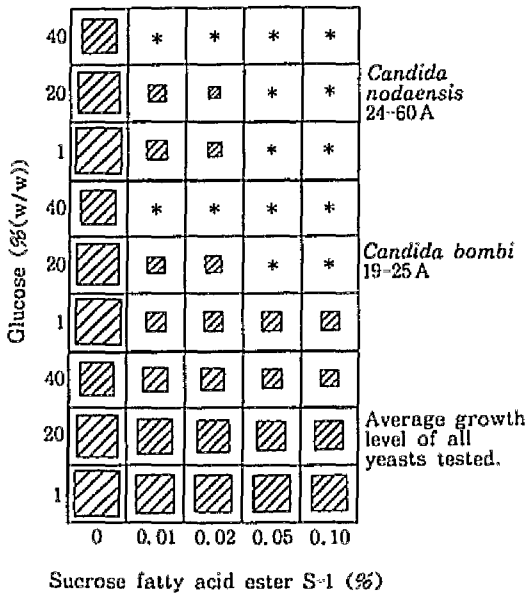


Fig.IV-2 Effects of concentrations of sucrose fatty acid ester S-1 on its inhibitory effects on the growth of yeasts

The area of ▨ shows the growth level of yeasts under different concentrations of sucrose fatty acid ester S-1. "*" shows "no growth". Growth levels of *Candida nodaensis* 24-60 A and *Candida bombi* 19-25 A, the growth of which was strongly inhibited by sucrose fatty acid ester S-1, and average growth level of all yeasts tested were shown.

40% glucose by 0.01% S-1. Thus, there were some differences in concentration dependence of antimicrobial effect among the tested surfactants.

The effects of 0.1% surfactants on the growth of 33 yeast strains are shown in Table IV-2. The growth inhibition indices of different surfactants on the growth of each strain were similar at each glucose concentration, so total growth inhibition index was calculated for each strain at each glucose concentration. Table IV-3 shows the relationships between glucose concentration of media and total growth inhibition index of the tested strains. In the presence of 1% glucose, the total growth inhibition indices of most strains were below 10, while it increased with the increase in glucose concentration. Higher antimicrobial effects at a higher glucose concentration may be due to that cell envelopes damaged by surfactants can not tolerate to a higher osmotic pressure. These results indicate that surfactants are

Table IV-3 Relationship between glucose concentration and inhibitory effect of surfactants on the growth of yeasts

| Total growth inhibition index | Number of strains | | |
|---|---|---|---|
| | 1% glucose | 20% glucose | 40% glucose |
| 0~10 | 26 | 17 | 1 |
| 11~15 | 4 | 8 | 4 |
| 16~20 | 1 | 5 | 8 |
| 21~25 | 1 | 2 | 9 |
| 26~30 | 0 | 1 | 7 |
| 31~35 | 0 | 0 | 4 |

effective for preventing the growth of yeasts in high-sugar foods.

The tested strains were divided into four types shown in Fig. IV-3, on the basis of the glucose concentration dependence of growth inhibition by surfactants. Among 32 tested strains, except for *C. glucosophila* 29-25A, which could not grow in 1% glucose, four strains belonged to type 1, 15 strains to type 2, 11 strains to type 3, and two strains to type 4. Representative strains belonging to each type are as follows; type 1: *Z. rouxii* 38-40A and *K. apis* 26-50A, type 2: *S. cerevisiae* 89-25A, *T. globosa* 26-40A, and *R. rubra* 70-25A, type 3: *S. pombe* 57-50B and *Rhodosporidium* sp. 65-25A, type 4: *P. membranaefaciens* 114-CA and *C. bombi* 19-25A. These four types did not relate to the groups of yeasts in taxonomy. This fact suggested that such differences among yeast species may not be caused by differences in taxonomic characteristics, but by those in structure of cell membranes.

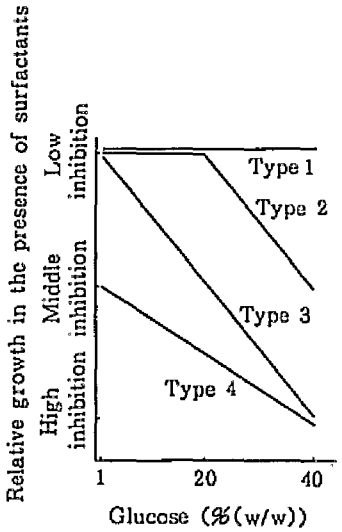


Fig.IV-3
Some types of glucose concentration dependence of inhibitory effects on the growth of yeasts

Fig. IV-4 compares the antimicrobial effects of nine surfactants at each glucose concentration. The effect was expressed with the average of growth inhibition indices of all tested strains. In 1% and 20% glucose, the effect of sucrose fatty acid ester S-1 was the highest and that of sucrose fatty acid ester S-3 followed. In 40% glucose, the effect of POE (47) laurylether was higher than those of S-1 and S-3. It is supposed that these differences in antimicrobial effect among surfactants may be due to differences in action mechanism on cell envelopes

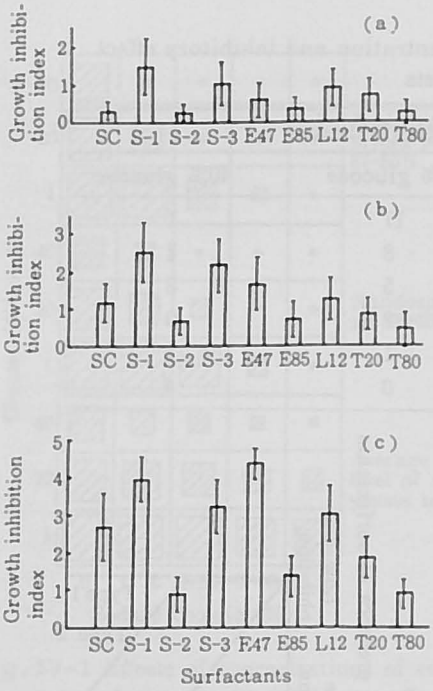


**Fig.IV-4**

Comparison of inhibitory effects of 9 surfactants on the average growth of all yeasts tested

(a) 1% glucose, (b) 20% (w/w) glucose, (c) 40% (w/w) glucose

SC: sodium cholate, S-1, S-2, and S-3: sucrose fatty acid ester, E 47: POE (47) lauryl ether, E 85: POE (85) nonylphenyl ether, L 12: POE (12) monolaurate, T 20: POE (20) sorbitan monolaurate, T 80: POE (20) sorbitan monooleate

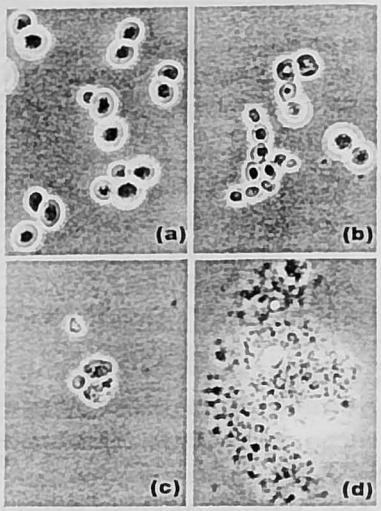


**Fig.IV-5**

Photographs of cells of *Torulaspora globosa* 26-40 A incubated in the presence or absence of surfactants

(a) 1% glucose medium, (b) 1% glucose medium supplemented with 0.5% sodium cholate, (c) 40% glucose medium supplemented with 0.1% sodium cholate, (d) 20% glucose medium supplemented with 0.1% sucrose fatty acid ester S-1

of yeasts. Among three sucrose fatty acid esters, those with higher HLB (S-1 and S-3) showed higher effects. This suggests that the solubility of surfactants might be also an important factor for determining their effects.

Cells of *T. globosa* 26-40A grown in the presence or absence of surfactants were observed using an optical microscope (Carl Zeiss, West Germany). Fig. IV-5(a) shows intact cells in the absence of surfactants. In 1% glucose medium supplemented with 0.5% sodium cholate and 40% glucose medium supplemented with 0.1% sodium cholate, distorted or almost broken cells were observed (Fig. IV-5(b,c)). In 20% glucose medium supplemented with 0.1% sucrose fatty acid ester S-1, cells were completely broken (Fig. IV-5(d)). Similar results were obtained in the presence of other surfactants. These observations showed that surfactants might act on the cell envelopes of yeasts and weaken their structure.

## IV-2. Effect of environmental gas composition

### IV-2-1. Effect of high concentrations of carbon dioxide

High concentrations of carbon dioxide has been already reported to show an inhibitory effect on the growth of fungi (8). The growth of some species including *Aspergillus restrictus*, *Penicillium cyclopium*, *Cladosporium* sp., and *Wallemia* sp. is significantly depressed by 10-30% carbon dioxide. The author investigated the effect of high concentrations of carbon dioxide on the growth of sugar-tolerant yeasts.

#### MATERIALS AND METHODS

*Strains*. Six isolates shown in Fig. IV-6 were used.

*Incubation*. Yeast cells preincubated on YM agar were one-point-inoculated onto YM agar plates. The plates were incubated at 25°C for one week in a chamber containing 100% carbon dioxide or gas mixtures composed of 0, 30, 50, or 80% carbon dioxide, 20% oxygen, and nitrogen. The diameter of colonies was measured and growth area was calculated.

#### RESULTS

As shown in Fig. IV-6, the growth rates of yeasts decreased with the increase in concentration of carbon dioxide. However, some strains belonging to such species as *C. tropicalis* and *S. cerevisiae* could grow even in 100% carbon dioxide. Thus, the effect of high concentrations of carbon dioxide against the growth of yeasts was not high enough to completely inhibit the growth of all species.

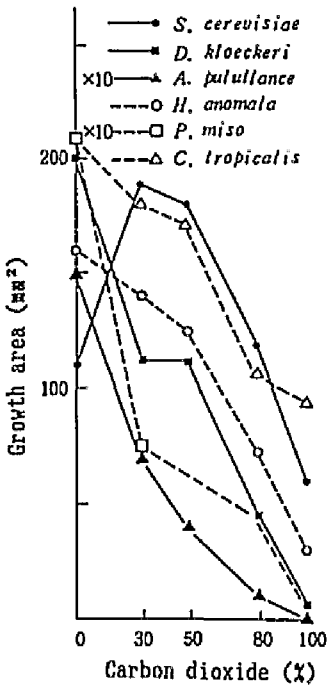


Fig. IV-6 Effect of concentration of carbon dioxide on the growth of yeasts

## IV-2-2. Effect of a low concentration of oxygen

Oxygen-absorber, which can easily decrease the concentration of oxygen in packages, is particularly effective for preventing growth of fungi and oxidation of oils, so widely used for food preservation. However, it is reported that oxygen-absorber is not effective for preventing the spoilage of foods by yeasts because of the ability of most yeasts to grow under anaerobic conditions (9). The effects of a very low concentration of oxygen obtained by oxygen-absorber on the growth of some sugar-tolerant yeasts were investigated at different $a_w$.

### MATERIALS AND METHODS

*Strains.* Five strains belonging to fermentative species, *H. anomala* 150-40A, *S. cerevisiae* 89-25A, *T. delbrueckii* 154-CA, *Z. rouxii* 88-40A, and *C. tropicalis* 118-40A were used.

*Preparation of media.* Six kinds of agar media containing 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and 29, 37, 43, 48, 53, or 57% (w/w) glucose as an $a_w$-controlling solute were prepared. These media had $a_w$ of 0.95, 0.93, 0.91, 0.89, 0.86, and 0.83, respectively.

*Incubation.* One hundred µl of cell suspensions with a concentration of $10^4$ cells/ml were inoculated onto agar plate media (30 x 50 mm). The plates were packaged together with an oxygen-absorber for 100 ml oxygen (Powdertech. Co., Ltd.) or without it, using γ-sterilized pouches (80 x 220 mm) of polyvinylidenechloride-coated oriented polypropylene / conventional polypropylene (KOP/CPP). The samples were stored at 25°C for 8 weeks.

*Measurement of volume of pouches, gas composition in headspace, and microbial count.* The volume of pouches was measured by putting pouches into water in a 1-liter measuring cylinder. Gas composition in headspace was measured using a gas chromatograph (Shimadzu GC-3AH, detector: TCD) equipped with two columns of molecular-sieve 5A and activated charcoal. The microbial count of yeasts was measured by the plating method after crushing the agar media using a stomacher (Lab-blender 400, Gunze Sangyo, Inc.).

### RESULTS AND DISCUSSION

Figs IV-7 - IV-11 show the changes in the microbial count of five yeast strains during storage. The growth rates and the maximum microbial counts of all the strains decreased with the

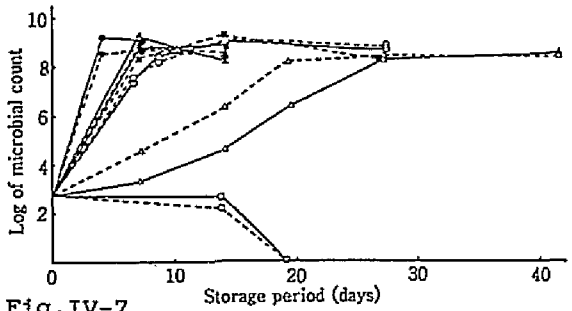


Fig. IV-7

Changes in microbial counts of *Hansenula anomala* 150-40A.

—— : control, ---- : with oxygen-absorber.
● : Aw 0.95, ▲ : Aw 0.93, ■ : Aw 0.91,
○ : Aw 0.89, △ : Aw 0.86, □ : Aw 0.83

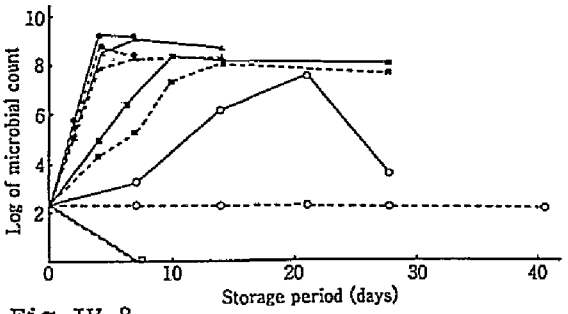


Fig. IV-8

Changes in microbial counts of *Saccharomyces cerevisiae* 89-25A.

—— : control, ---- : with oxygen-absorber.
● : Aw 0.95, ▲ : Aw 0.93, ■ : Aw 0.91,
○ : Aw 0.89, △ : Aw 0.86, □ : Aw 0.83

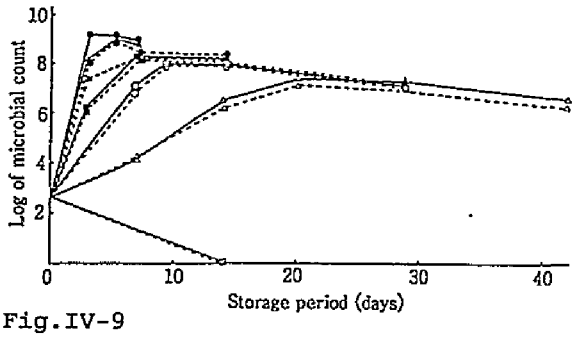


Fig. IV-9

Changes in microbial counts of *Torulaspora delbrueckii* 154-CA.

—— : control, ---- : with oxygen-absorber.
● : Aw 0.95, ▲ : Aw 0.93, ■ : Aw 0.91,
○ : Aw 0.89, △ : Aw 0.86, □ : Aw 0.83.

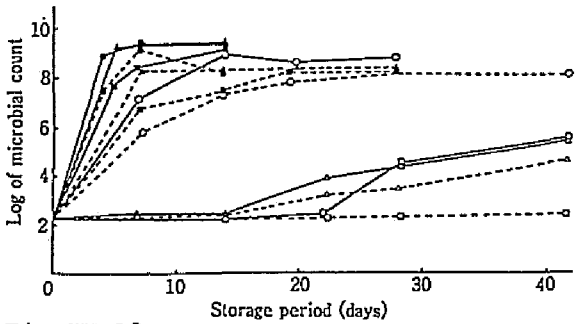


Fig. IV-10

Changes in microbial counts of *Candidia tropicalis* 118-40A.

—— : control, ---- : with oxygen-absorber.
● : Aw 0.95, ▲ : Aw 0.93, ■ : Aw 0.91,
○ : Aw 0.89, △ : Aw 0.86, □ : Aw 0.83.

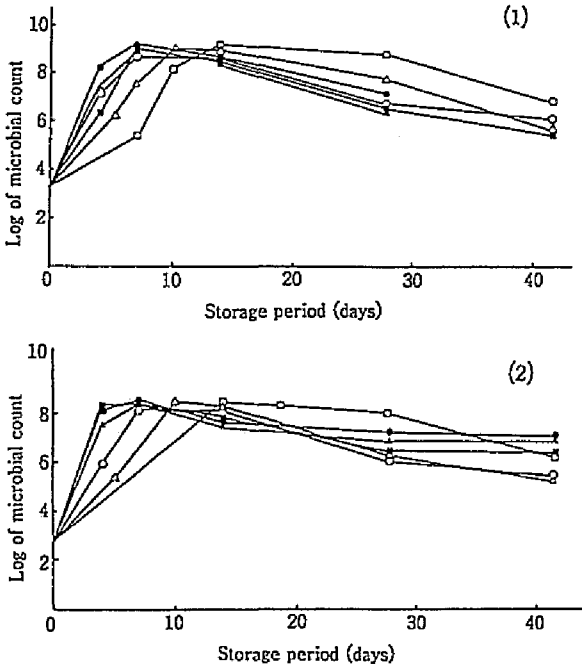


Fig. IV-11

Changes in microbial counts of *Zygosaccharomyces rouxii* 88-40A.

(1) control, (2) with oxygen-absorber.
● : Aw 0.95, ▲ : Aw 0.93, ■ : Aw 0.91,
○ : Aw 0.89, △ : Aw 0.86, □ : Aw 0.83.

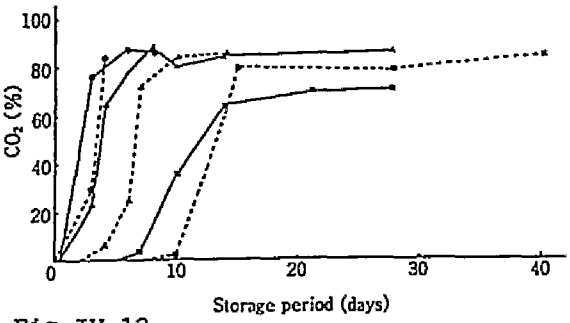


Fig.IV-12
Changes in concentration of carbon dioxide in the pouches containing *Saccharomyces cerevisiae* inoculated media.
—— : control, ---- : with oxygen-absorber.
● : Aw 0.95, ▲ : Aw 0.93, ■ : Aw 0.91,

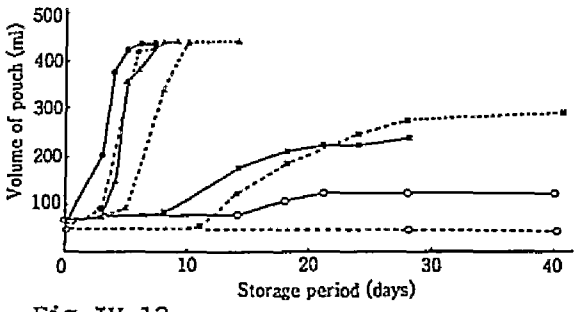


Fig.IV-13
Changes in volume of pouches containing *Saccharomyces cerevisiae* inoculated media.
—— : control, ---- : with oxygen-absorber.
● : Aw 0.95, ▲ : Aw 0.93, ■ : Aw 0.91,
○ : Aw 0.89,

decrease in $a_w$ of media. Among the five strains, the growth of *S. cerevisiae* 89-25A (Fig. IV-8) and *C. tropicalis* 118-40A (Fig. IV-10) was slightly depressed by oxygen-absorber. Relatively high effects of oxygen-absorber were observed near the minimum $a_w$ for their growth. On the other hand, the growth rates and the maximum microbial counts of *H. anomala* 150-40A (Fig. IV-7), *T. delbrueckii* 154-CA (Fig. IV-9), and *Z. rouxii* 88-40A (Fig. IV-11) were not affected by oxygen-absorber.

The concentration of oxygen in pouches containing oxygen-absorber reached less than 0.01% within 2 hr after packaging. The concentration of carbon dioxide dramatically increased in the logarithmic stage of the growth of yeasts (Fig. IV-12). When the microbial count reached the maximum, the concentration of carbon dioxide was about 80%. The volume of pouches increased as the concentration of carbon dioxide increased (Fig. IV-13). These results indicated that the swelling of pouches was caused by the generation of carbon dioxide.

The results in this study showed that the inhibitory effect of oxygen-absorber on the growth of fermentative sugar-tolerant yeasts was very low beyond expectation.

It was reported that the oxidation reduction potentials for the growth of yeasts ranged from -160 to +90 mV and considerably varied with species. It is supposed that the potentials might affect their growth rates in the presence of oxygen-absorber.

## IV-3. Effect of ethanol vapor - low oxygen combination method

Ethanol is widely used for food preservation because of its antimicrobial effect (11) and safety. In recent years, ethanol vapor generator, containing powdered ethanol and gradually generating ethanol vapor, has been developed and used mainly for preventing the growth of fungi in intermediate moisture foods.

It is known that a larger amount of ethanol is needed for preventing the growth of yeasts, particularly in high moisture foods, because of higher ethanol-tolerance of yeasts. A large amount of ethanol is responsible for the deterioration of food flavors.

In order to increase the antimicrobial effect of ethanol, the effects of an ethanol vapor- low oxygen combination method on the growth of sugar-tolerant yeasts were studied at different $a_w$ in comparison with those of ethanol vapor.

### MATERIALS AND METHODS

*Strains.* The five strains tested in IV-2-2 were used.

*Preparation of media.* Five kinds of agar media containing 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and 12, 32, 44, 49, or 53% (w/w) glucose were prepared. These media had $a_w$ of 0.97, 0.94, 0.91, 0.89, and 0.86, respectively.

*Incubation.* Preincubated cells were inoculated onto the media as described in IV-2-2. The media were packaged together with an ethanol vapor generator or an oxygen absorption type- ethanol vapor generator containing 0.5 g ethanol (Freund Industry Co., Ltd.) or without them, using sterilized pouches (80 x 220 mm) of KOP/CPP film. They were incubated at 25°C for 8 weeks.

*Measurement of ethanol concentration and gas composition in headspace and microbial count.* Ethanol concentration in headspace of pouches was measured using a gas chromatograph (Shimadzu GC-14A) equipped with a Porapack Q (type N, 80/100 mesh) column. Gas composition in headspace and microbial count were measured as described in IV-2-2.

### RESULTS AND DISCUSSION

Ethanol concentrations in headspace of pouches containing an ethanol vapor generator or an oxygen absorption type- ethanol vapor generator were equilibrated (1.0-1.5 % (v/v)) within 24 hr after packaging. In the samples where yeasts grew, the concentrations of ethanol and carbon dioxide in headspace increased with the increase in microbial count and the swelling

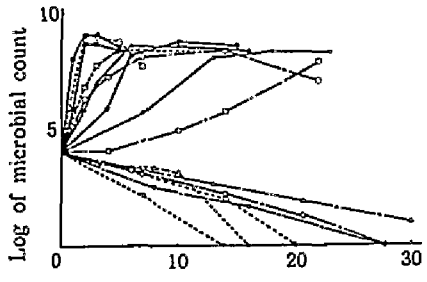


Fig.IV-14 Changes in microbial counts of *Hansenula anomala* 150-40 A during storage

——: Control, ----: Ethanol vapor generator, ——: Oxygen absorption type-ethanol vapor generator

●, ○: Aw 0.97, ■, □: Aw 0.94, ▲, △: Aw 0.91, ◆, ◇: Aw 0.89, ▼, ▽: Aw 0.86

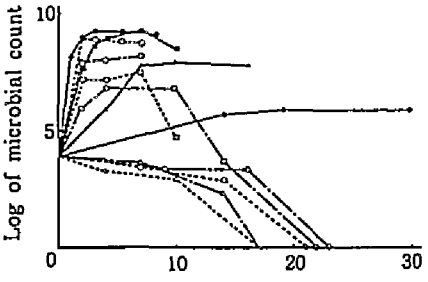


Fig.IV-15 Changes in microbial counts of *Saccharomyces cerevisiae* 89-25 A during storage

——: Control, ----: Ethanol vapor generator, ——: Oxygen absorption type-ethanol vapor generator

●, ○: Aw 0.97, ■, □: Aw 0.94, ▲, △: Aw 0.91, ◆, ◇: Aw 0.89, ▼, ▽: Aw 0.86

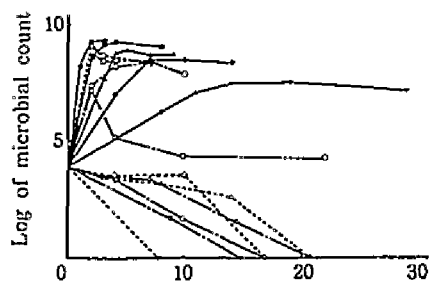


Fig.IV-16 Changes in microbial counts of *Torulaspora delbrueckii* 154-CA during storage

——: Control, ----: Ethanol vapor generator, ——: Oxygen absorption type-ethanol vapor generator

●, ○: Aw 0.97, ■, □: Aw 0.94, ▲, △: Aw 0.91, ◆, ◇: Aw 0.89, ▼, ▽: Aw 0.86

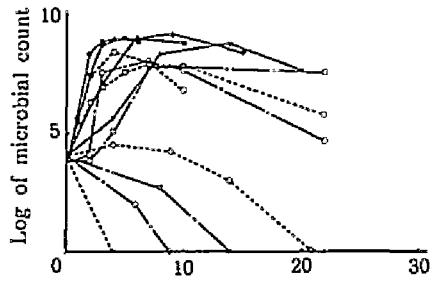


Fig.IV-17 Changes in microbial counts of *Zygosaccharomyces rouxii* 88-40 A during storage

——: Control, ----: Ethanol vapor generator, ——: Oxygen absorption type-ethanol vapor generator

●, ○: Aw 0.97, ■, □: Aw 0.94, ◆, ◇: Aw 0.89, ▼, ▽: Aw 0.86

of pouches was observed. Oxygen concentration in headspace of pouches containing an oxygen absorption type-ethanol vapor generator reached less than 0.01% within 3 hr after packaging.

The changes in microbial count of *H. anomala* 150-40A, *S. cerevisiae* 89-25A, *T. delbrueckii* 154-CA, *Z. rouxii* 88-40A and *C. tropicalis* 118-40A were shown in Figs IV-14 - IV-18. In all strains, the decreases in growth rate and/or maximum microbial count were observed in the presence of ethanol vapor generator or oxygen absorption type- ethanol vapor generator. Both ethanol vapor method and ethanol vapor- low oxygen combination method

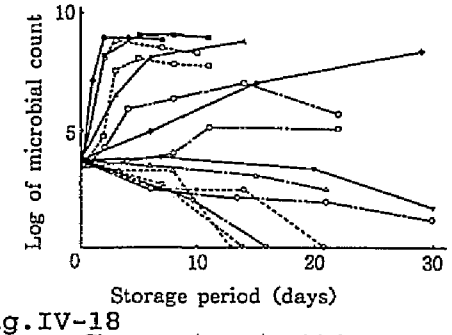


Fig.IV-18

Changes in microbial counts of *Candida tropicalis* 118-40 A during storage

——: Control, ----: Ethanol vapor generator, ——: Oxygen absorption type-ethanol vapor generator

●, ○: Aw 0.97, ■, □: Aw 0.94, ▲, △: Aw 0.91, ◆, ◇: Aw 0.89, ▼, ▽: Aw 0.86

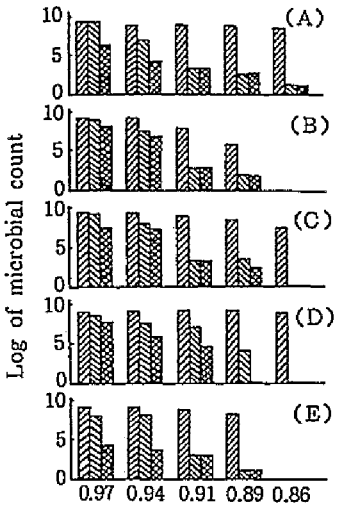


Fig.IV-19

Comparison of inhibitory effects of two types of ethanol vapor generator on the growth of yeasts at different water activities

Microbial counts at the time when microbial count in control at each water activity reached the maximum level were compared. (A) *Hansenula anomala* 150-40 A, (B) *Saccharomyces cerevisiae* 89-25 A, (C) *Torulaspora delbrueckii* 154-CA, (D) *Zygosaccharomyces rouxii* 88-40 A, (E) *Candida tropicalis* 118-40 A

▨: Control, ▧: Ethanol vapor generator, ▩: Oxygen absorption type-ethanol vapor generator

gave higher inhibitory effects on the growth of the tested strains at lower $a_w$, and the combination method was more effective than ethanol vapor method.

Fig. IV-19 compares the microbial counts at the time when those in the absence of both types of ethanol vapor generator reached the maximum at each $a_w$. In most strains, the inhibitory effects of the combination method were the highest at $a_w$ 0.94 and the growth at lower $a_w$ was inhibited by only ethanol. The effects of the combination method were particularly high against the growth of *Z. rouxii* 88-40A and *C. tropicalis* 118-40A.

The fact that the combination method is more effective for depressing the growth of yeasts compared with the method using only ethanol vapor indicates that a smaller amount of ethanol is required by the combination method for preventing the spoilage of foods by yeasts, resulting in the solution of the odor problem.

## IV-4. Effect of natural antimicrobial compounds

### IV-4-1. Effect of allylisothiocyanate

Many of spices are known to have antimicrobial effects (12-15) and they have been used for food preservation. Mustard is one of the spices with high antimicrobial activities. The activity of mustard was found to be due to its major pungent compound, allylisothiocyanate (AIT) (16). Kanemaru and Miyamoto (17) confirmed this by comparing the antibacterial activity of brown mustard extract and synthesized AIT against putrefactive bacteria.

The inhibitory effects of AIT on the growth of sugar-tolerant yeasts were tested in the form of indirect addition because of its volatility.

#### MATERIALS AND METHODS

*Strains.* The five strains tested in IV-2-2 were used.

*Preparation of volatile mustard extract.* Oil was extracted with ethanol from *Brassica juncea* seeds and concentrated under a reduced pressure. Volatile mustard extract was prepared by dissolving the oil containing 25 mg of AIT in 0.5 g of palm oil.

*Storage test using volatile mustard extract.* Preparation of media, incubation of yeasts, and measurement of microbial count were done as described in IV-3, except for an incubation period of 2 weeks. The concentration of AIT in headspace was measured using a gas chromatograph (Shimadzu GC-14A) equipped with a DEGS (15%, 80/100 mesh) column.

*Determination of minimum inhibitory concentration (MIC) of AIT.* One hundred µl of cell suspensions with a concentration of $10^5$ cells/ml were inoculated onto YM agar plates. Filter papers supplemented with 100 µl of corn oil containing different concentrations of AIT were put on the inside of caps of petri dishes. The caps were covered with inoculated agar plates and the petri dishes were packaged into sterilized pouches (110 x 110 mm) of ethylene-vinylalcohol copolymer (EVOH) /CPP film. The MIC was the lowest concentration which prevented visible growth after one week at 25°C.

#### RESULTS AND DISCUSSION

Fig. IV-20 shows the changes in microbial count of *H. anomala* 150-40A during storage. This strain grew at all tested $a_w$ of 0.97 to 0.86 in the absence of volatile mustard extract, while the

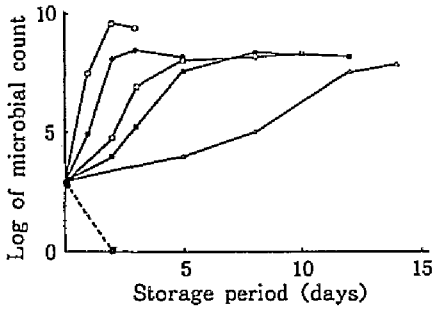


Fig.IV-20

Changes in microbial counts of *Hansenula anomala* 150-40 A during storage

---- : with volatile mustard extract,

—— : control,

○ : Aw 0.97, ● : Aw 0.94, □ : Aw 0.91, ■ : Aw 0.89, △ : Aw 0.86

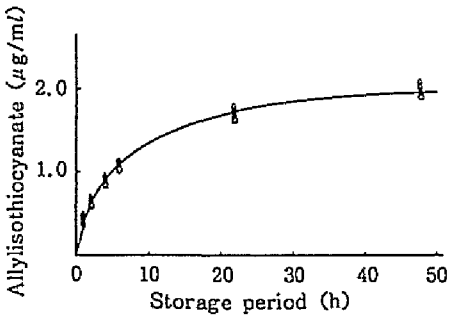


Fig.IV-21

Changes in concentration of allyl-isothiocyanate in headspace during storage

○ : Aw 0.97, ● : Aw 0.94, □ : Aw 0.91, ■ : Aw 0.89, △ : Aw 0.86

Table IV-4

Effects of volatile mustard extract on the growth of yeasts isolated from high-sugar foods

| Strain | Musturd extract vapor | Log of viable count*1 | | | | |
|---|---|---|---|---|---|---|
| | | Aw 0.97 | Aw 0.94 | Aw 0.91 | Aw 0.89 | Aw 0.86 |
| *Hansenula anomala* 150-40 A | − | 9.66 | 8.16 | 8.07 | 5.44 | 2.81 |
| | + | ND*2 | ND | ND | ND | ND |
| *Saccharomyces cerevisiae* 89-25 A | − | 9.18 | 7.56 | 6.80 | 3.32 | ND |
| | + | ND | ND | ND | ND | ND |
| *Torulaspora delbrueckii* 154-CA | − | 9.58 | 8.01 | 8.65 | 7.54 | 7.04 |
| | + | ND | ND | ND | ND | ND |
| *Zygosaccharomyces rouxii* 38-40 A | − | 8.32 | 7.30 | 8.99 | 7.05 | 9.05 |
| | + | ND | ND | 3.30 | ND | ND |
| *Candida tropicalis* 118-40 A | − | 8.61 | 7.71 | 7.80 | 5.11 | 2.80 |
| | + | ND | ND | ND | ND | ND |

*1 Values after 2 days incubation at Aw 0.97 and 0.94, after 5 days incubation at Aw 0.91 and 0.89, and after 10 days incubation at Aw 0.86.

microbial count decreased below the limit of detection within 2 days after packaging in the presence of it. Similar results were obtained for other four strains as shown in Table IV-4.

The changes in AIT concentration in headspace of pouches were shown in Fig. IV-21. The concentration of AIT reached the maximum (2 μg/ml) at all $a_w$ within 24 to 48 hr after packaging. The amounts of AIT dissolving in media were very small. This fact indicated that the antimicrobial effect of volatile mustard extract was due to AIT vapor in headspace.

The MICs of AIT for five yeast strains were determined as

Table IV-5 Minimum inhibitory concentrations of allylisothiocyanate on yeasts isolated from high-sugar foods

| Strain | Minimum inhibitory concentration (ng/ml) |
|---|---|
| *Hansenula anomala* 150-40 A | 14.8 |
| *Saccharomyces cerevisiae* 89-25 A | 9.5 |
| *Torulaspora delbrueckii* 154-CA | 7.4 |
| *Zygosaccharomyces rouxii* 38-40 A | 2.8 |
| *Candida tropicalis* 118-40 A | 10.6 |

shown in Table IV-5. *H. anomala* was the most resistant to AIT and *Z. rouxii* the least resistant of the yeast tested.

The odor threshold of AIT was estimated to be around 3 ng/ml by a sensory test. In fact, the AIT odor was detected when pouches containing the MICs of AIT were open. Therefore, it is needed to solve this odor problem by developing methods for removing or masking the odor, in order to use volatile mustard extract for depressing the growth of yeasts.

### IV-4-2. Effect of silver ions

Some metal ions are known to have antimicrobial effects. In particular, silver ions and copper ions have high activity (18, 19) and they have been used for many years in the disinfection of water.

Silver-zeolite has been developed as an agent to mix into plastic films because of high antimicrobial activity of silver ions and high safety (20, 21). Zeolites are hydrated crystalline aluminosilicates, and have charge-compensating cations such as sodium ion, responsible for its ion-exchange property, in the crystalline structure. Silver ions exchange for sodium ions with high efficiency and selectivity, resulting in the formation of silver-zeolite with different silver exchange ratios. Plastic films supplemented with silver-zeolite have antimicrobial activity on the surface of the inside of pouch or sheet. It is supposed that they are highly safety because the amounts of silver ions moving into foods or foodstuffs are very small.

Using silver-zeolite samples, the effects of silver ions on the growth of sugar-tolerant yeasts were investigated.

#### MATERIALS AND METHODS

*Strains.* Three strains, *S. cerevisiae* 89-25A, *Z. rouxii* 86-A, and *C. tropicalis* 118-40A, were used.

*Silver-zeolite samples.* Silver-zeolite powder used were proportionated by Shinagawa Fuel Co., Ltd. The silver exchange ratios and silver contents of zeolite powder are shown in Table IV-6.

Table IV-6 Silver exchange ratio and silver content of zeolite powder used.

| Zeolite powder | Silver exchange ratio (%) | Silver content (wt %) |
|---|---|---|
| Ag- 0 | 0.0 | 0.0 |
| Ag- 5 | 4.8 | 3.0 |
| Ag-10 | 9.8 | 5.6 |
| Ag-40 | 40.1 | 20.6 |
| Ag-70 | 66.8 | 31.8 |
| Ag-90 | 90.4 | 37.8 |
| Ag-95 | 96.4 | 38.5 |

*Determination of MIC of silver-zeolite.* Five ml of the cultures of yeast strains were inoculated into 100 ml of YM broth supplemented with different concentrations of each silver-zeolite powder. The MIC was the lowest concentration which prevented visible growth after incubation in a rotary shaker at 30°C for 24 hr. The effects of concentration of components in broth on the MIC of silver-zeolite were investigated using YM broth and its diluted solutions with different dilution ratios. The MICs of silver-zeolite under anaerobic condition were compared with those under aerobic condition by incubating the strains on YM agar plates supplemented with different concentrations of silver-zeolite in the presence and absence of oxygen-absorber.

*Determination of released silver ions.* To measure the concentration of silver ions released from silver-zeolite into broth, YM broth supplemented with silver-zeolite was left at 30°C for 24 hr, followed by filtration of the broth through a membrane filter with a pore size 0.1-0.2 μm (Nihon Millipore Kogyo K.K.). Silver ion concentration in the supernatant was determined using an atomic absorption spectrophotometer (Nippon Jarrel Ash, Model AA-1).

## RESULTS AND DISCUSSION

The MICs of silver-zeolite with different silver exchange ratios for *Z. rouxii* 86-A and the concentrations of silver ions in broth at the MICs were shown in Table IV-7. The MIC of each

Table IV-7 Inhibitory effect of different silver-zeolite on the growth of *Zygosaccharomyces rouxii*

| Zeolite powder | MIC (ppm) | $Ag^+$ concentration in broth at MIC (ppm) |
|---|---|---|
| Ag- 5 | 128 | 4.6 |
| Ag-10 | 128 | 4.6 |
| Ag-40 | 32 | 4.8 |
| Ag-70 | 8 | 2.8 |
| Ag-90 | 8 | 1.8 |
| Ag-95 | 8 | 1.6 |

Table IV-8 Effect of oxygen on MICs of silver-zeolite for *Zygosaccharomyces rouxii*

| Zeolite powder | MIC under aerobic condition (ppm) | MIC under anaerobic condition (ppm) |
|---|---|---|
| Ag- 5 | 128 | 128 |
| Ag-10 | 128 | 128 |
| Ag-40 | 32 | 32 |
| Ag-70 | 16 | 16 |

silver-zeolite decreased as its silver exchange ratio increased. Almost the same MICs were obtained for other two strains. Silver-zeolite with higher exchange ratios showed the antimicrobial effect at lower concentrations of released silver ions, about 2 ppm. These results suggested that the antimicrobial effect of silver-zeolite with high exchange ratios might be due to silver ions directly taken from silver-zeolite into yeast cells as well as those released into broth.

Incubation in the absence of oxygen did not affect the MICs of silver-zeolite as shown in Table IV-8. This indicates that the antimicrobial effect of silver-zeolite is due to the action of silver ion itself, although there is a theory that active oxygen is produced by silver-zeolite and responsible for the effect (22).

It was supposed that the antimicrobial effect of silver-zeolite might be affected by the concentration of components in broth such as amino acids and sulfur compounds. Fig. IV-22 shows the relationships between the MIC of silver-zeolite and the dilution fold of YM broth. The MICs of silver-zeolite for three yeast strains decreased almost linearly as the dilution fold of the broth increased, i. e., the concentration of components

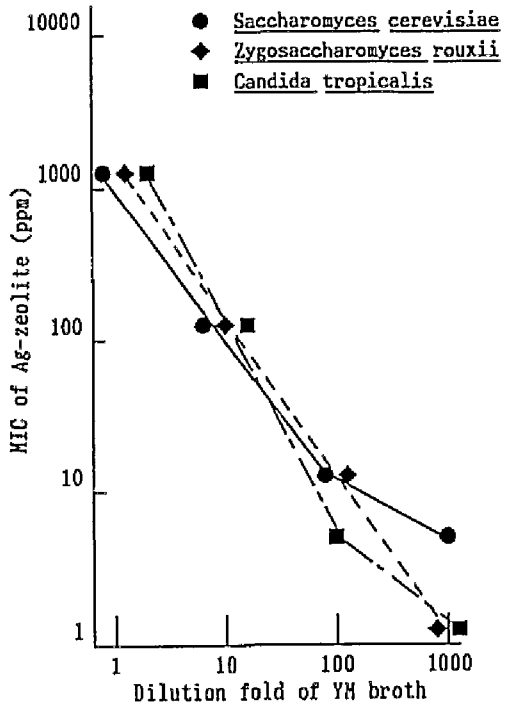


Fig.IV-22 Effect of concentration of broth components on MIC of Ag-zeolite for yeasts

decreased. In the presence of a high concentration of components, they might combine with silver ions, resulting in the requirement of a high concentration of silver ions in broth. Therefore, it is supposed that silver-zeolite may not be effective for preserving foods rich in nutrients, packed at a small relative contact area.

Silver ions have a very wide antimicrobial spectrum and show almost the same activities against a number of microorganisms including bacteria, yeasts, and fungi (23). This fact suggests that the action of silver ions might be the damage to the components found in all microorganisms and the inhibition of their functions. It is supposed that silver ions may be taken directly or indirectly from silver-zeolite into cells of microorganisms and adsorb proteins in cell membranes or organelles, resulting in the inhibition of the essential metabolisms in cells by interfering with enzymes.

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## SUMMARY

CHAPTER I

Yeast strains were isolated from 265 samples of high-sugar foods and related materials, and 99 representative strains were identified. Fifty-four strains of ascosporogeneous yeasts were identified as 12 species belonging to eight genera. Thirty-nine strains of asporogenous yeasts were identified as 26 species belonging to five genera. These results indicated that sugar-tolerant yeasts distributed in taxonomically wide ranges. Five undescribed strains isolated from brown sugar, from sponge cake, and from flowers were identified as four new species, *Candida glucosophila, Candida dulciaminis, Candida floricola, and Candida vaccinii*. One undescribed yeast-like fungus was also isolated from orchid nectar and the new genetic and species name *Sympodiomycopsis paphiopedili* to accommodate the fungus was introduced.

CHAPTER II

Minimum water activities ($a_w$) for the growth of 35 isolates were determined using three kinds of sugars (glucose, fructose, and sucrose) and sodium chloride as $a_w$-controlling solutes. Most species showed the highest minimum $a_w$ for growth in NaCl-media and about half the lowest minimum $a_w$ for growth in sucrose-media. One strain of *Zygosaccharomyces rouxii* had minimum $a_w$ for growth as low as 0.67 in a fructose-medium. Such nutrients as inositol and casamino acid, and preincubation in the presence of a high concentration of sugars decreased minimum $a_w$ for growth.

CHAPTER III

The sugar-tolerant yeasts intracellularly accumulated polyols, mainly glycerol and D-arabitol. It was suggested that these polyols could contribute to the osmoregulation of sugar-tolerant yeasts and that glycerol might be an important intracellular solute for the osmoregulation. Three forms of erythrose reductase were purified and characterized from an extremely sugar-tolerant *Aureobasidium* sp. mutant having high erythritol-producing activity.

It was shown that the characteristics of cell membranes of sugar-tolerant yeasts were significantly different among yeast species, based on the results on the effects of glucose concentration of cultivation media on the lipid composition, $H^+$-pumping activity, and fluidity and rigidity of cell membranes.

CHAPTER IV

An ethanol vapor- low oxygen combination method was found to be one of the most effective techniques for depressing the growth of sugar-tolerant yeasts, which could be applied for the preservation of intermediate moisture foods. Some surfactants including sucrose fatty acid esters showed higher inhibitory effects on the growth of the yeasts with the increase in glucose concentration. The effect of carbon dioxide increased as the concentration increased, while that of a very low oxygen concentration obtained by oxygen-absorber was very low. Allylisothiocyanate vapor and silver ions tested in the form of silver-zeolite showed high antimicrobial effects, although further investigations are necessary for the practical application to food preservation.

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